Behaviour of *Listeria monocytogenes* in New Zealand fresh apple supply chains

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

By

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Declaration of Originality

This is to certify that to the best of my knowledge; the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

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Abstract

Listeria monocytogenes (*L. monocytogenes*) is a pathogenic bacterium. It can be a market access issue if found on fresh produce like apples, especially in countries with "zero tolerance" for bacteria like the USA. New Zealand has an export-based economy, and apples are an essential produce item, accounting for almost a quarter of the export value of fresh produce. Although New Zealand has recorded no outbreak or recalls for apples, the potential exists for *L. monocytogenes* to be present on apples through the soil, water, and packhouse. As there is no kill-step for *L. monocytogenes* on apples, a rigorous assessment of potential food safety risks from apples is needed across the supply chain.

The literature review found knowledge gaps in understanding the survival of *L. monocytogenes* under dynamic temperature conditions, which is a reality in the international or domestic supply chain. The thesis investigated: (i) The effect of dynamic temperatures on *L. monocytogenes* in the international supply chain, (ii) The effect of dynamic temperatures on *L. monocytogenes* in the domestic supply chain, (iii) The effect of storage temperatures on the survival of three lineages of *L. monocytogenes* on apples, (iv) The effect of commercial storage temperatures on the survival and attachment of three lineages of *L. monocytogenes* on two apple cultivars.

The dynamic temperatures for the international supply chain through sea freight suggested that for both open and closed calyx apple cultivars, *L. monocytogenes* did not grow but declined throughout the study. In simulated dynamic temperature studies for both the USA and Europe, *L. monocytogenes* survived better in the calyx than on the skin of the apple ($P < 0.05$), regardless of whether inoculated into open or closed calyx apple cultivars. Shelf-life studies at 20°C showed no significant difference from dynamic temperatures in reducing *L. monocytogenes* ($P > 0.05$). Destructive postharvest assessments like fruit firmness (Kgf), and soluble solids (SSC, °Brix) did not correlate with the survival of *L. monocytogenes* ($P >$

0.05). The result indicated that irrespective of time and temperature, the international sea-freight supply chain does not result in the growth of *L. monocytogenes*.

The dynamic temperatures were recorded in different years for every step of the domestic supply chain, i.e., transportation, distribution centre (DC), retail outlet, and display cabinet. The recorded temperatures of the supply chain were simulated in the laboratory trials, using open and closed calyx cultivars inoculated with a seven-strain cocktail of *L. monocytogenes*. The bacteria were inoculated in the calyx and on the body of both the apple cultivars. The results showed that, regardless of the season, *L. monocytogenes* declined when the dynamic temperatures of transportation trucks, DCs, retail outlets, and display cabinets were applied. As in the international supply chain, *L. monocytogenes* survived better in the calyx than on the body ($P < 0.05$) for the domestic supply chain. Although no growth was observed for *L. monocytogenes*, inactivation kinetics suggested that the resistant bacteria were still present at the end of each study.

In a challenge study, the survival of three lineages of *L. monocytogenes* was investigated at the critical static temperatures of apple supply chains, i.e. 0.5, 2, 6, 20°C. High and lower inoculums were used in the calyx and body to evaluate the carrying capacity of *L. monocytogenes* of apples. At the study's conclusion, no growth was observed for high and lower inoculums at any temperature. A comparative quantitative study was performed using two quantitative methodologies: MPN (Most Probable Number) and qPCR (Quantitative Polymerase Reaction). The results from both methods showed that lineage III of *L. monocytogenes* died much quicker than lineages I and II.

A scanning electron microscopy (SEM) study was performed at different temperatures to understand the attachment of the three lineages of *L. monocytogenes*. The results demonstrated that lineage I and II creates more biofilms than lineage III for different apple

cultivars and at different temperatures (0.5, 2, 6, and 20°C). However, all the lineages can potentially make biofilms under ideal temperatures and environmental conditions. *L. monocytogenes* form more robust biofilms in the calyx than on the body of the apple, possibly due to symbiotic fungal-bacterial interactions.

In conclusion, the research has proved that dynamic temperatures in the international and domestic apple supply chains did not result in the growth of *L. monocytogenes* for various apple cultivars. The study also demonstrated that *L. monocytogenes* of lineage I and II has better survival and biofilm-making capacity under commercially critical apple supply chain temperatures than lineages III. The comparative quantitative challenge study using MPN and qPCR methods explored options for a faster and more accurate way to quantify *L. monocytogenes* and found higher bacterial concentrations in the qPCR than MPN method, which could be due to nucleic acids originating from dead cells in qPCR interfering with the final results. This thesis provides helpful insights into the survival of *L. monocytogenes* on different apple cultivars that can be used to develop effective risk mitigation strategies for the fresh apple supply chain.

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I designed the studies with the co-authors, analysed the data and wrote the drafts.

The content provided in Chapter 3, Chapter 4, and Chapter 5 have also been developed and are presented as manuscripts with the intention of submitting them for publication. In these chapters, I designed the studies with the co-authors, analysed the data and wrote the drafts.

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Contents

List of Figures

List of Tables

List of Abbreviations

1-MCP	1-Methylcyclopropene
ANOVA	Analysis of Variance
ARC	Australian Research Council
BLEB	Buffered *Listeria* Enrichment Broth
CA	Controlled Atmosphere
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
Csp	Cold Shock Protein
DC	Distribution centre
DNA	Deoxy ribonucleic acid
E. coli	*Escherichia coli*
ESEM	Environmental Scanning electron microscopy
EPS	Exopolymer Substances
EU	European Union
FAO	Food and Agriculture Organization
FSANZ	Food Standards Australia New Zealand
Fig.	Figure
GFSI	Global Food Safety Initiative
HACCP	Hazard Analysis Critical Control Points
ITTC	Industrial Transformation Training Centre
LI	Lineage I
LII	Lineage II
LIII	Lineage III
L. monocytogenes	*Listeria monocytogenes*
NZ	New Zealand
PCR	Polymerase Chain Reaction
PFR	Plant and Food Research
qPCR	Quantitative Polymerase Chain Reaction
RH	Relative humidity

RNA	Ribonucleic acid
RTE	Ready-to-eat
SEM	Scanning Electron Microscopy
SPI	Starch Pattern Index
TSAYE	Tryptic Soy Agar with yeast extract
TSB	Tryptic soy broth with yeast extract
USA	United States of America
WHO	World Health Organization
USDA	The United States Department of Agriculture
USFDA	The United States food and drug administration

Chapter 1. Literature review

1.1 Background

The awareness of eating fresh fruits and vegetables for good health and their ever-increasing demand lead to a high level of fresh produce production worldwide (Olaimat and Holley, 2012) (van Dyk et al., 2016). The expansion of the fresh produce industry is characterised by increased production, a centralised distribution system, expanded global trade of fresh produce, and surveillance efficiency (Lynch et al., 2009). The growing production of fresh produce worldwide also leads to a complex supply chain (Wadamori et al., 2017).

Despite the beneficial health effects of fresh produce, there is growing awareness concerning its microbial food safety (Strawn et al., 2011). Increased demand for fresh produce is linked to increased human infections and foodborne outbreaks in recent years because fresh produce could serve as a host for several foodborne pathogens (Al-Kharousi et al., 2016; Kuan et al., 2017), resulting in foodborne illnesses. Foodborne illness is a disease that occurs when the food and water are contaminated by bacteria, viruses, and parasites are consumed (Ali et al., 2021; Camino Feltes et al., 2017). In the past 30 years, as the food production and consumption have increased, foodborne disease associated with fresh produce has increased as well (Mukherjee et al., 2006), e.g. the outbreaks related to fresh produce in the United States increased from 0.7% in the 1970s to 46% in 2014 (van Dyk et al., 2016). In 2009, and 2010 in the European Union (EU), 4.4% and 10% of the foodborne illnesses and outbreaks were linked to fruits, vegetables and related products, respectively (Calvin et al., 2006). These foodborne outbreaks have severe consequences for public health and have a significant economic impact (Calvin et al., 2006). For example, in the United States in 2013, it was estimated that the economic burden due to foodborne outbreaks were between $4.8 to $36.6 billion (Sandra et al., 2015).

Since 1990, possibly due to increased surveillance as well as actual increases, there has been an increase in reported fresh produce-related outbreaks worldwide linked to foodborne pathogens (Denis et al., 2016). Significant outbreaks happened in the past decade (Table 1.1).

Table 1.1: Outbreaks associated with fresh produce (2013-2021).

Year/country	Produce	Pathogen	Cases	Hospitalisations	Deaths
2021	Packaged salad	*L. monocytogenes*	18	16	2
2019	Romaine	*Escherichia coli* O157:H7	167	85	-
2018	Rockmelon	*L. monocytogenes*	19	-	6
2016/Australia	Rockmelon	*Salmonella* Hvittingfoss	97	-	-
2016/Australia	Pre-package lettuce	*Salmonella* Anatum	144	-	-
2016/UK	Imported salad	*E.coli* O157	161	60	2
2016/USA	Packaged Salads	*L. monocytogenes*	19	19	1
2015/Australia	Imported frozen strawberries	Hepatitis A virus	19	-	-
2015/USA	Imported cucumber	*Salmonella* Poona	900	204	6
2014/USA	Pre-packaged caramel apples	*L. monocytogenes*	32	31	6

2014/USA	Mung bean sprouts	*L. monocytogenes*	5	5	2
2014/New Zealand	Fresh vegetables (exact source remains unknown)	*Yersinia pseudotuberculosis*	334	65	-
2014/UK	Lettuce, cucumber	Entero invasive *E. coli* O96	50	-	-
2014/UK	Salads	*Salmonella* Singapore	4	-	-
2014/USA	Raw clover sprouts	*E. coli* O121	19	8	-
2014/USA	Coriander	*Cyclospora cayetanensis*	304	7	-
2013/USA	Bean sprouts	*Salmonella* Enteritidis	87	27	-
2013/USA	Imported cucumber	*Salmonella*	84	17	-
2013/USA	Imported pomegranate seeds	Hepatitis A virus	165	69	-
2013/USA	Salad mix	*Cyclospora cayetanensis*	631	50	-
2013/USA	Imported cucumber	*E. coli* O157: H7	33	11	-

Table from (Wadamori et al., 2017). Outbreaks after 2018 added later.

1.2 Introduction

Fresh produce that is ready to eat (RTE) is generally not heated or cooked before consumption. Thus, the fresh produce should be produced free of pathogens or decontaminated by other means before consumption. Failing to do so could result in foodborne illness and potentially lead to food recalls, leading to significant economic loss

(Luchansky et al., 2017). The total food recall cost from all food-borne pathogens were estimated to be $10 billion annually in the USA alone (Ostroff, 2018).

All the fresh produce potentially can get contaminated with human pathogens, as evident by the diversification of types implicated in outbreaks, e.g. cucumbers that had been rarely associated with any outbreak before 2012 are now linked to a high number of *Salmonella* cases (Sharma et al., 2017). On the other hand, *Salmonella* outbreaks were high in tomatoes before 2011, and now are rare (Sreedharan et al., 2014).

In parallel to the diversification, there has been a shift in the association between produce and pathogen (Murray et al., 2017), e.g., apples. In apples, the pathogen of concern was *E. coli* O157: H7 (Alegre, Abadias, Anguera, Usall, et al., 2010). However, *Listeria monocytogenes (L. monocytogenes)* caused an outbreak from caramel apples (Salazar et al., 2016).

Several factors contributed to the diversification of pathogens and fresh produce linked to outbreaks, including increased consumption, an ageing population, and the fresh produce supply chain (Murray et al., 2017). However, the most crucial factor has been the advent of affordable DNA sequencing that can detect pathogens and identify/ link the borne illness cases to products (Taboada et al., 2017). Before the advent of DNA sequencing, the source provenance of foodborne pathogen was 20%, compared to 70% since then (Emond-Rheault et al., 2017). If the produce is contaminated, it has more chance of being detected (Murray et al., 2017). This shift in the ease of pathogen detection potentially means a need to understand the pathogen of concern. If we understand the pathogen, there are chances to mitigate the risks associated with them in the supply chain.

Currently, the most common foodborne pathogens that are linked to fresh-produce outbreaks are *L. monocytogenes, Salmonella enterica,* and *E. coli* O157: H7 (Timmons et al., 2018). *L.*

monocytogenes is of specific concern (Angelo et al., 2017). From 2011 to 2015, there have been 17 recalls in the USA due to *L. monocytogenes* (Luchansky et al., 2017).

1.3 ***Listeria monocytogenes***

L. monocytogenes is a member of the genus *Listeria*, and it can be found in rural (or urban in that matter) areas such as manure, soil, and water. This bacterium is pathogenic and can cause a rare disease called listeriosis (Zhu et al., 2017), which mainly affects immune-compromised people, e.g., pregnant women and their unborn children, people aged >65, and infants and toddlers (Zhu et al., 2017). The United States Food and Drug Administration (USFDA) mentioned that 2500 people suffer from listeriosis annually (Ostroff, 2018). Compared to other diseases caused by foodborne pathogens, listeriosis is relatively rare but has a high fatality rate of up to 30% (Scallan et al., 2011). Listeriosis mainly occurs above 100 CFU/g (Chen et al., 2002), although that depends on the genetic lineage, serovars (a group of bacteria characterised by specific set of antigens), and the immunocompromised nature of the consumer (FSANZ, 2013). Most of the foodborne outbreaks from *L. monocytogenes* have been reported in the USA, Canada, Europe, Australia and New Zealand (Todd and Notermans, 2011).

This pathogen is known to grow at temperatures from -1.5 to 45°C (Uchima et al., 2008) and variable pH ranging from 4 - 9.6 (Santos et al., 2019). Depending on the type of fresh produce, if the fresh produce is not stored at its desired temperature, this pathogen can multiply (Yeni et al., 2016).

Based on the variable gene content, *L. monocytogenes* consists of four phylogenetic lineages, lineage I, II, III and IV. Lineage I is commonly associated with most outbreaks and clinical cases (Orsi et al., 2011). Lineage II is mainly found in food processing facilities and food products (Kabuki et al., 2004). This might be because lineage II strains have higher recombination rates than lineage I, leading to adaptation in various ecological niches (den

Bakker et al., 2008; Pirone-Davies et al., 2018). Lineage III and IV are rare and predominantly found from the animal sources (Wiedmann et al., 1997), however, some uncommon occurrences of human listeriosis cases due to lineage III suggested not to undervalue the foodborne exposure to this strain (Roberts et al., 2006; Wiedmann et al., 1997). Hence understanding every lineage is crucial as they all have the capacity to be pathogenic. Understanding every lineage could be done in the form of a cocktail of all the lineages (assuming all the lineages are present in one sample), or individually.

1.3.1 Infectious dose of L. monocytogenes

Listeriosis can cause illness in a healthy and immunocompromised population, however, it depends on the virulence of the bacterial strain (FSANZ, 2013), and the amount of dose ingested, and or genetic lineage of *L. monocytogenes* (Quereda et al., 2021).

There is no concrete data available that can establish the contamination levels required for listeriosis (Quereda et al., 2021). Even so, a dose of 10^7 to 10^9 colony-forming units (CFUs) in healthy hosts, and 10^5 to 10^7 CFUs in immunocompromised individuals is considered to be infective for listeriosis (Pouillot et al., 2016; Quereda et al., 2021). Based on the above information about the dosage levels in the literature, it becomes crucial to understand the bacteria at those concentrations.

To control *L. monocytogenes* in food, regulatory agencies have required food industries to develop hazard analyses at critical control point (HACCP) programmes and have strictly regulated *L. monocytogenes* contamination of food (Archer, 2018). The uncertainty of minimal infectious dose, the severity of the disease and virulence differences observed among strains means immunocompromised people including the elderly, and pregnant women should avoid eating food likely to contain high amounts of *L. monocytogenes*. For healthy populations, it is advised to handle high-risk foods carefully, and to store them at low temperatures (Quereda et al., 2021).

1.3.2 Major outbreaks due to L. monocytogenes

In 2011, a multistate outbreak due to *L. monocytogenes* in the USA involved the whole cantaloupe melons, resulting in 147 confirmed cases and 33 deaths. This cantaloupe outbreak has been the deadliest foodborne disease outbreak in the USA's history (Martinez et al., 2016). An investigation found that the bacteria were present in the packing facility. However, those strains were not detected in soil samples from the fields where the cantaloupes were grown (McCollum et al., 2013). It was found that the whole cantaloupes had been contaminated by the processing plant and equipment (Martinez et al., 2016). In Australia in 2018, a *L. monocytogenes* outb*reak* in cantaloupes resulted in six deaths (Guardian, 2018).

In 2014/2015, caramel apples contaminated with *L. monocytogenes* resulted in 7 deaths (Chen et al., 2016). Bidart Brothers of Bakersfield, California, recalled 'Granny Smith' and 'Gala' apples because environmental testing revealed contamination with *L. monocytogenes* at the firm's apple-packing facility. The state and federal investigators found *L. monocytogenes* on polishing brushes, drying brushes, a packing line drain, inside a wooden bin and on an automatic packing line (Beecher, 2016). Also, three patients who got sick during this outbreak did *not* eat caramel apples but rather fresh cut or whole apples, which indicates that apples in whole or fresh-cut formats infected with *L. monocytogenes* can cause human illnesses (Angelo et al., 2017; Marus et al., 2019). It was also unexpected that *L. monocytogenes* could persist on whole/fresh cut apples to the point of consumption. This outbreak was the first-ever *L. monocytogenes* outbreak in apples and highlighted that fresh apples could be a risk for *L. monocytogenes* contamination (Angelo et al., 2017). This outbreak underlined the importance of *L. monocytogenes* and its ability to survive in various conditions on fresh produce.

In 2017, an apple company in the USA recalled 'Gala', 'Fuji', Honeycrisp and 'Golden Delicious' apples due to *L. monocytogenes* (FDA, Food and Drug Administration 2020). In

another recall, another Michigan apple-producing company recalled four varieties of apples due to the presence of *L. monocytogenes* (FDA, Food and Drug Administration 2019).

As *L. monocytogenes* in the above-mentioned outbreaks proved fatal, and lead to market recalls, it showed the importance of understanding the pathogen of concern which can cause illnesses and market access issues. The literature behind these outbreaks also showed the lack of information available on the behaviour of *L. monocytogenes* on fresh apples, either in the packhouse or during storage. Overall, these outbreaks also showed the need to understand the knowledge of lineages in apple supply chain and its nature of infection.

1.4 Apples

Apples are one of the most consumed fruit in the world. In 2016, 89.33 million tonnes of apples were produced (Statista, 2016). New Zealand has an agriculture and horticulture based economy, where apples get exported to all the major countries of the world (MBIE, 2017). New Zealand's apple industry has the highest productivity globally, averaging 65 metric tonnes per hectare per annum. In 2017 and 2018, 'The World Apple Review' ranked New Zealand top in international competitiveness among 33 apple exporting countries, scoring across 23 criteria (Freshfacts, 2020).

Nutritionally, one serving of apple provides ~ 95 calories, 1 g protein, 25 g carbs and 3 g fibre. Apples help fight against cardiovascular disease, Type 2 diabetes, and weight issues (Boyer and Liu, 2004).

To preserve the fruit quality, most apple cultivars are stored at 0.5°C. To slow the maturation processes even further, oxygen and carbon dioxide concentrations are set in controlled atmosphere (CA) storage, typically at 1% oxygen and 1–2% carbon dioxide, depending on the cultivar. Once fruit has cooled, it needs to be kept at these low temperatures even during shipping. As far as humidity is concerned, maintaining the humidity from 90 – 95% will

reduce weight loss. Some varieties are held in plastic liners to maintain the humidity at high levels (Palmer, 2008).

1.4.1 Apple as a model for challenge study

After the 2014-2015 caramel apple incident (Angelo et al., 2017), the majority of food safety research on tree-fruit has been apple-centric. However, there is still a lack of understanding of the bacterial behaviour in the apple supply chain, especially to understand the prevalence of *L. monocytogenes*.

In a study to understand the prevalence of *L. monocytogenes* in apple packing facilities, especially in and around the storage rooms, samples were collected from the facility before and after the sanitation practices. It was found that microbial contamination can enter the packhouse on fruit (e.g. through picking bins, soil on the fruit) vehicles, cleaning equipment and worker foot traffic (Walter et al., 2016). Although the prevalence of *L. monocytogenes* is less than other *Listeria spp*., *L. monocytogenes* contamination can happen at various steps in the production line (Ishani et al., 2017).

Upon leaving packhouse storage, fruit is exposed to potential contaminants from equipment, vehicles, distribution centre and retail stores. This increases the risk of microbial contamination, growth and survival of the microorganisms involved (Portman et al., 2002). Effective cold management is crucial to apple's preservation, quality, and safety; hence, research to establish the risk during these phases is essential.

The shelf-life of an apple after harvest is much longer than that of many other fruits; often, it reaches the consumer six months after harvest. Thus, information on the capacity of *L. monocytogenes* to grow in apples stored for more than three months is of great importance (Macarisin et al., 2019), especially for an export-based country like New Zealand that is distant to many markets.

Although New Zealand has recorded no outbreak or recall of apples for *L. monocytogenes*, there is a possible food safety risk associated with the apple supply chain.

Apples are considered to be a fruit of high risk under the microbial risk criteria Category 2 (Portman et al., 2002).

Table 1.2: Microbial risk categories for fruit.

Category 1*	Category 2*	Category 3	Category 4	Category 5
Skin eaten, Rough skin	Skin eaten, smooth surface	Skin generally not eaten	Skin not eaten, the crop grown on ground	Skin not eaten, crop grown above ground
Stonefruit	Grape	Citrus	Melons	Avocado
Berry fruits	Tomato		Pineapple	Mango
	Stone fruit			Banana
	Apple and Pear			Kiwifruit
	Persimmon			Passionfruit
	Nectarine			Feijoa
	Plum			Lychee

*****Category 1 and 2 are high-risk category. Table from (Portman et al., 2002).

These categories mentioned above are only recommendations and guidelines based on current knowledge. The most common guidelines for acceptance are currently < 100 CFU/g for *L. monocytogenes*. However, other countries, such as the USA, Austria, and Italy, require the total absence of *L. monocytogenes* in 25 g of foods (FSANZ, August 2014).

The United States Food and Drug Administration (FDA) Food Safety Modernization Act (FSMA) 2011 'produce safety rule' established, for the first time, science-based minimum standards for the safe growing, harvesting, packing and holding of fruit and vegetables grown for human consumption (FDA, Food and Drug Administration 2011). In Australia and New Zealand, food safety in fresh produce is self-regulated through best practice guidelines and

industry codes of conduct. As mentioned before, New Zealand has an export-based economy, and *L. monocytogenes* could pose a serious food safety market access risk to the New Zealand apple industry, and the potential economic and reputational impact of such an event is significant. Implementing proactive control measures to avoid future *Listeria* outbreaks is required and could only be accomplished after examining the behaviour of *Listeria* throughout the supply chain (Zhu, 2015).

Fresh produce can become contaminated with *L. monocytogenes* from manure, cross-contamination during harvesting, postharvest handling and storage, distribution, storage in the market, consumer handling, and consumption. Therefore, it becomes imperative to quantitatively evaluate the risk of *L. monocytogenes* (Ding et al., 2013).

Very little is known about the effect on apple food safety of international, domestic supply chains and storage, all of which typically happen for exports. These effects are crucial from New Zealand's perspective, as storage, transportation, and retail are the three critical phases of the supply chain that have been overlooked in food safety research.

1.5 Review of the international apple supply chain and its effect on *L. monocytogenes*

1.5.1 Apples:

Figure 1.1 represents an overview of a simplistic typical cold chain in the fresh produce supply, especially for apples. The cold chain usually starts from harvesting and ends in a retail store. The total duration of any cold chain varies and depends on the product; it can be as short as a few hours and can be longer than a few months (Mercier et al., 2017).

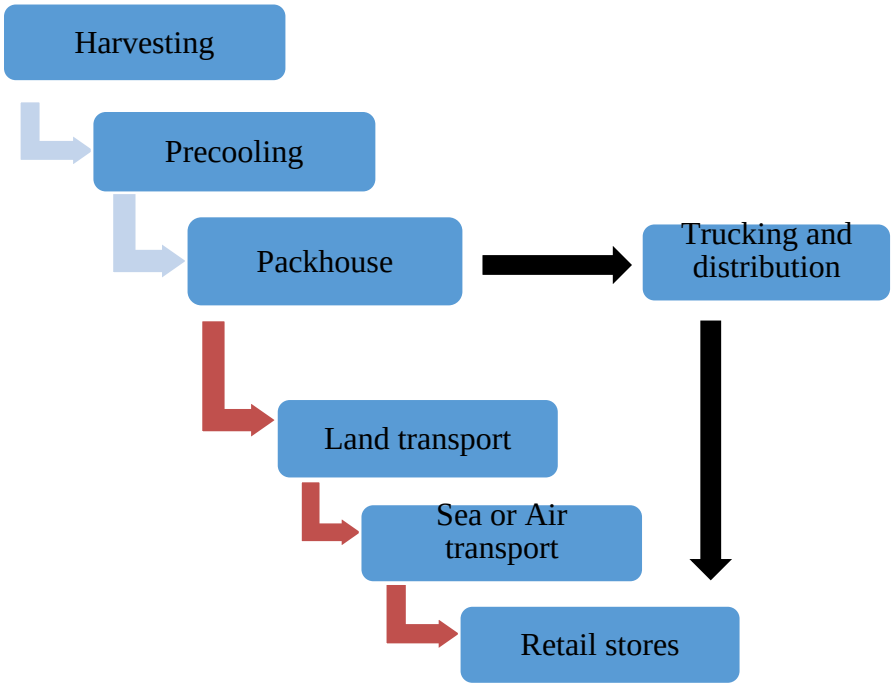

Figure 1.1: Overview of flow diagram of a typical apple supply chain, where orange arrows shows international and black arrows show domestic supply chain.

As suggested in Fig. 1.1, the apple supply chain has many stages, which means there are many potential sources of microbial contamination and cross-contamination. Every step of the supply chain could affect bacterial growth, e.g. the apple type or cultivar, process conditions, and prior temperature management, storage, and transportation can all affect the fate of microorganisms on fresh produce (Zagory, 1999). Temperature is a significant factor that can slow down or increase the growth of bacteria on fresh produce surfaces in the supply chain (Danyluk et al., 2012). A critical issue with temperature in a supply chain is that low temperatures does not reduce or kill bacteria; they are only forced into a latent state. Additionally, pathogens such as *L. monocytogenes* can grow at refrigeration temperatures (Danyluk et al., 2012). Hence, research to understand and establish the risk during the different supply chain step is essential.

Postharvest handling and transport conditions in the apple supply chain play a considerable role in determining the fate of any bacteria (Sibomana et al., 2017). If non-refrigerated

vehicles are used, the variable cold chain and substantial temperature differences throughout the transport can influence the quality of the fresh produce and lead to opportunistic bacteria growing on the fresh produce (Sibomana et al., 2017). In tomatoes, the temperature differences in the non-refrigerated trucks result in the condensation on the surface of tomatoes, allowing rots to grow (Sibomana et al., 2017). This research showed the importance of low temperatures and their impact on controlling microbial growth.

Sheng *et al.* (2017) in their study of conventional 'Fuji', organic and conventional 'Granny Smith' apples, dip-inoculated fruit with *L. monocytogenes* to establish ~3.5 and 6.0 $\log_{10}$ CFU/apple and stored them at 1, 4, 10 and 22°C for up to 12 weeks. The different storage temperatures were sampled at 1, 4, 7, and 14 days for short-term storage for all temperatures and 2, 4, 8, and 12 weeks for long-term storage at 1, 4, and 10°C. For 2-week short-term storage, *the L. monocytogenes* population on organic 'Granny Smith' at 1, 4, or 10°C was reduced by 0.2-0.3 $\log_{10}$ CFU/apple. For the long term 12-week storage at 1, 4, and 10°C, the *L. monocytogenes* counts on organic 'Granny Smith' decreased by 0.5-1.5 $\log_{10}$ CFU/apple for both the inoculation levels. Conventional 'Granny Smith' and 'Fuji' apples had a similar survival pattern with 0.8-2.0 $\log_{10}$ CFU/apple reduction over 12 weeks of cold storage. At two weeks of storage at 22°C, both the apple varieties behaved similarly with 0.5-1.7 $\log_{10}$ CFU/apple reduction, regardless of inoculation levels. Growers in the packhouse industry assume that *L. monocytogenes* on apples do not grow in long term cold storage. However, this study showed that cold temperature is insufficient to significantly reduce *L. monocytogenes* on fresh apples (Sheng et al., 2017). The storage study mentioned above was carried out under static (fixed) temperatures under controlled conditions. We do not know whether these results would be similar under dynamic (variable) temperature conditions, such as occurs in domestic or international supply chains. Also, it is unclear whether apples'

physiological condition and maturity play any role in the persistence of *L. monocytogenes* on apples.

In another study, 'Red Delicious', 'Granny Smith', and 'Fuji' apples were inoculated with *L. monocytogenes* strains and stored for 160 days. It was found that *L. monocytogenes* survived better in the calyx than on the skin for all three cultivars (Macarisin et al., 2019). In another study in 'Gala', 'Granny Smith' and 'Honeycrisp' apples, three apple cultivars were dip inoculated with a cocktail of strains of *L. monocytogenes* and air-stored under a controlled atmosphere (1.5% O_2, 1.5% CO_2) stored for 12 weeks (Ryser et al., 2019). There was no significant difference between *L. monocytogenes* survival in the air and CA stored apples (Ryser et al., 2019). Also, *Listeria* concentrations were still quantifiable in some samples after 7 months of storage, despite the population decreasing. The study was done over two years, and the bacterial survival was greater in year 1 ($P < 0.05$) (Ryser et al., 2019). All the studies discussed above were performed at long-term low temperatures. However, as mentioned before, whether similar results would be obtained in realistic temperatures in the apple supply chain, where the temperature is ever-changing is unclear.

In another study on the growth of foodborne pathogens on minimally processed apples stored at 20 and 25°C, *Escherichia coli* O157: H7, *Salmonella,* and *Listeria innocua* increased by more than 2 $\log_{10}$ over a 24 h period on fresh-cut apple plugs. Of the three tested, *L. innocua* was the only organism able to grow at 25°C (Alegre, Abadias, Anguera, Oliveira, et al., 2010). It is important to note that there is a vast difference between whole and minimally processed fresh produce (fresh-cut) e.g. apples. Fresh-cut apples, during processing exposes the cytoplasm and provides the bacteria with nutrient rich environment, resulting in facilitation of rapid microbial growth (Qadri et al., 2015).

Other studies in the literature also used *L. monocytogenes* surrogates on apples e.g. a study by (Sheng et al., 2018) also suggested that *L. innocua* decreased in number by 2.5 – 3 $\log_{10}$ CFU/apple, but persisted in apples for 30 weeks of cold storage. *L. innocua* resembles *L. monocytogenes* closely in genetic makeup. However, there are few differences between these two organisms, e.g., *L. innocua* grows faster than *L. monocytogenes* below 8°C. Also, the lag time of *L. monocytogenes* is more than *L. innocua*, below 8°C. (Omac et al., 2015). Furthermore, *L. innocua* surrogate strains cannot precisely mirror the behaviour of *L. monocytogenes* under stress conditions (O'Bryan et al., 2006). The most significant difference between *L. monocytogenes* and *L. innocua* is the absence of virulence gene cluster (prfA, plcA, hly, mpl, actA) in *L. innocua* (Milillo et al., 2012). Hence, I therefore believe that it is important to study the fate of pathogenic bacteria *Listeria monocytogenes* rather than observing the behaviour of *L. innocua* as a surrogate bacterium along the apple supply chain.

1.5.2 Other fruits and vegetables:

Like apples, other fruits and vegetables need low temperature for the extended shelf life. However, appropriate knowledge of storage temperature and humidity can also help producers delay the growth of foodborne pathogens in the fresh produce as well (Gil et al., 2015). Avocados are gaining popularity worldwide. However, the contamination problems with *L. monocytogenes* are ever-increasing (Cabrera-Díaz et al., 2022) due to insufficient research done to understand the effect of *L. monocytogenes* for long-term storage. A comparative study between *L. monocytogenes* and *Salmonella* in avocados was performed at 5°C for 48 days After the study, *L. monocytogenes* showed a lower rate of decline than *Salmonella* (Cabrera-Díaz et al., 2022). This research could be further used for risk assessment studies in the future.

Gil et al. (2015) stated the importance of storage and temperature of fresh leafy vegetables, mentioning that *L. monocytogenes* could survive cold temperatures below 3.8°C (Gil et al., 2015).

Generally, the storage temperature for cantaloupes is around 3 - 4°C (EFSA, 2014). In cantaloupes, like other fresh produce, temperature plays a vital role in the growth of *L. monocytogenes*. A study found that the population of *L. monocytogenes* (6 $\log_{10}$ CFU/ cantaloupe) on stem scars was significantly higher ($P < 0.05$) by 1-2 $\log_{10}$ CFU/ cantaloupe when stored at 25°C on day 1 compared to 4°C or 10°C (Nyarko et al., 2016). For fresh-cut cantaloupes, fresh-cut cubes were inoculated with *L. monocytogenes* and *S. enterica* (~ 4 $\log_{10}$ CFU/g) at 4, 8 and 12°C and stored for one week. *Salmonella* cell counts were increased by -0.26, 1.39 and 2.23 $\log_{10}$ units at 4, 8 and 12°C, respectively, whereas *L. monocytogenes* increased by 0.75, 2.86 and 4.17 $\log_{10}$ units. Controlling temperature is critical for controlling the growth of foodborne bacterial pathogens (Huang et al., 2015).The growth of any human pathogens on acidic fresh produce is considered limited, but the growth of *E.coli* O157: H7, *Salmonella choleraesuis*, and *L. innocua* were studied on minimally processed peaches at temperatures 5, 10, 20 and 25°C. At 10°C, only *L. innocua* grew more than 1 $\log_{10,}$ and it was the only pathogen to grow at 5°C (Alegre, Abadias, Anguera, Usall, et al., 2010). This shows the importance of a rigid cold chain to avoid the persistence of the foodborne pathogens on peaches.

In whole mangoes, the population of *L. monocytogenes* increased at all the temperatures from 0.1-1.4 $\log_{10}$ CFU/mango when the fruit was inoculated with a *L. monocytogenes* cocktail (6 $\log_{10}$ CFU/mango) and stored at 12, 20 and 30°C (Friedrich and Danyluk, 2017).

Jalapeno peppers can carry foodborne pathogens as well. To understand the growth of human pathogens, jalapenos were inoculated with five strains of *L. monocytogenes*, *E.coli* O157: H7,

and *S. enterica* on the intact external surface, injured outer surface and the intact internal cavity of jalapenos that were then stored for 14 days at 7 and 12°C. For every scenario, *L. monocytogenes* was capable of growth and survival. This result shows the significance of storing and finding an ideal temperature for jalapenos so that *L. monocytogenes* could not grow (Huff et al., 2012). *L. monocytogenes* can persist in a frozen food environment as well. However, with fresh produce, the levels of viable *L. monocytogenes* on refrigerated and intact produce like strawberries at the point of consumption depend on the initial contamination load, length of storage and the temperature (Flessa et al., 2005). A study was done to determine the significant differences in growth/survival differences in *L. monocytogenes* on intact or cut, fresh and frozen inoculated strawberries. Cut or intact strawberries were spot inoculated with *L. monocytogenes* at 10^8 and 10^6 CFU. Intact berries got approximately 0.6, and 1.2 $\log_{10}$ reductions at high and low inoculum, respectively, was observed after the 1 hr drying period. A decrease of 1.4 and 3.3 $\log_{10}$ cycles per intact sample were observed over 48 hours. For high and low inoculum, respectively, when stored at 24°C. At 4°C, 3 $\log_{10}$ reduction was observed for whole strawberries over a seven day period. The population of cut surfaces did not change much. After four weeks of storage, the population declined by 0 to 1.2 $\log_{10}$ only for frozen produce. The results showed that *L. monocytogenes* could survive on fresh and frozen strawberries (Flessa et al., 2005).

In fresh-cut and frozen foods, *L. monocytogenes* could also pose a human health risk for low moisture foods (LMFs) (Cuzzi et al., 2021). A challenge study was performed with *L. monocytogenes* on raisins and dried strawberries for 365 days at 4°C, which showed that *L. monocytogenes* persisted when the starting inoculum was 5 $\log_{10}$ CFU/g (Cuzzi et al., 2021).

1.6 Review of the domestic apple supply chain on the effect of *L. monocytogenes* and obtaining an awareness on the risks along distribution centres and grocery stores

1.6.1 Apples

Apples, including any fresh produce, are distributed from the packhouse to distribution centres (DC), and finally to the retail shops (Zhu et al., 2016). Three risk factors contributing to the contamination and growth of foodborne pathogens, including *L. monocytogenes* in retail environments, are a. time and temperature abuse b. cross-contamination c. improper cleaning and sanitation (Cutter, 2017).

Effective cold chain management is essential for preserving produce quality and food safety (Rediers et al., 2009). However, it is not always possible in the real world due to logistics and breaks in cooling which leads to temperature "abuse". Refrigeration is an essential part of maintaining food safety and the quality of foods. In a review, James et al. (2017) stated a loss of 40% of fresh produce when the food goes from farm to fork, showing the importance of refrigeration and cooling.

Temperature control is vital for the maintenance of fresh produce quality and shelf life (Smyth et al., 1998), including apples. Generally, depending on the cultivar, the optimal storage temperature for apples is 0.5°C. Apple temperature monitoring trials on 'Granny Smith' and 'Cripps pink' cultivar have found the produce had a temperature abuse of 57 containers out of 111 (51%) (Goedhals-Gerber et al., 2021). Food and Drug Administration (FDA) requires all refrigerated foods to be stored at 5°C or less to control the bacterial growth and microbial safety (Tian et al., 2013).

1.6.1a Steps of domestic apple supply chain postharvest

The primary sources of bacterial contamination of fresh produce occur in postharvest conditions than pre-harvest (Johnston et al., 2005). *L. monocytogenes* persist in the packinghouse and processing environment (Townsend et al., 2021). For example, out of 1437

samples collected in three commercial tree-fruit packhouse facilities, 17.5% were *L. monocytogenes* positive (Simonetti et al., 2021). After cooling and storage at the packhouse, fresh produce goes to the distribution centres, generally by refrigerated trucks. Trucking, distribution centres (DC) and grocery or retail stores are the key elements of a successful domestic supply chain.

Trucking is the standard mode of delivering fresh produce from one place to another (Pelletier et al., 2011). The trucks carrying fresh produce from either pre-packhouse or post-packhouse are regarded as the carrier of microbial contamination (Warriner and Hasani, 2020). E.g. In an outbreak from *L. monocytogenes* on cantaloupes (Buchanan et al., 2017), the trailer used to transport the fruit had previously been used to carry animals. Although the trailer was not linked to *L. monocytogenes*, it can be predicted that the risk still existed (Warriner and Hasani, 2020).

The DC is a critical part of many cold chain management systems. It provides the opportunity to sort and combine shipments received from many suppliers and to schedule their departure to supermarkets and smaller retail shops (Mercier et al., 2017). The DC is a critical control point in the cold chain management systems (Mercier et al., 2017).

The retail store is the last link of the cold chain infrastructure, where the recommended temperature should be maintained (Tokala and Mohammed, 2021). When the fresh produce reaches the grocery store, it is generally received at a coolstore (generally above 0°C), and placed on display either in refrigerated cabinet, a stand under air conditioning or at ambient temperatures. Time-temperature measurements indicate that the display cabinet temperature rises above the desired limit (Mercier et al., 2017).

Retail store is the primary place where most of the fresh produce is bought by household, restaurants or food-service outlets. If there is bacterial contamination in the produce from

retail store, it is fair to assume that contaminated produce capable of causing foodborne illness pass through these facilities (Townsend et al., 2021). There is limited information and data associated with contamination in the fresh produce supply chain, as it is complicated to assess the associated risk, especially from *L. monocytogenes*. Hence more research is needed to assess the persistence of *L. monocytogenes* at the domestic supply chain level (Townsend et al., 2021). Due to the complex and multistep nature of the domestic supply chain, every step has the potential for temperature abuse, which could contribute to cross-contamination and bacterial proliferation. Gathering enough quantitative data at every step of supply chain ensures the food safety of the fresh produce and helps mitigate any future risks. Additional studies evaluating *L. monocytogenes* prevalence at the domestic supply chain level are needed to determine the sources of bacterial contamination or proliferation (Townsend et al., 2021).

1.6.2 Other fruits and vegetables

Whether in retail or farmer's market, bacterial pathogens are present. 3.7% of 1372 fresh leafy vegetables and 1.8% of 1160 fresh-cut vegetables were contaminated with *L. monocytogenes* and *Salmonella* in Italy (Losio et al., 2015). Eleven of 30 retail grocery stores were found positive for *L. monocytogenes* in 2017 in seven states in the USA which suggested that retail environments could be one of the trigger points of *L. monocytogenes* contamination (Burnett et al., 2017). A study by (Vital et al., 2017) showed that a variety of fresh produce like bell pepper, carrot, lettuce, and tomatoes bought from open-air and supermarkets can act as a pool of pathogenic bacteria such as *E.coli* and *Salmonella* spp., which are harmful to the well-being of consumers (Vital et al., 2017).

In a study examining the presence of pathogens in the fresh produce from Florida (USA) farmer's market and supermarkets, and out of 401 different produce samples, *L.*

monocytogenes was detected in 3.9% and 2.6% of farmer's market spinach and leafy greens, respectively (Roth et al., 2017).

A microbial analysis of lettuce, cucumber, cabbage, and carrot, to detect *L. monocytogenes* in retail shops in Canterbury, New Zealand, and found lettuce to have the highest level of *L. monocytogenes* (4.2 $\log_{10}$ CFU/g), followed by cucumber (3.2 $\log_{10}$ CFU/g), cabbage (2.5 $\log_{10}$ CFU/g) and carrot (Zhu et al., 2016). In Salvador, Brazil, a total of 132 samples, including raw, frozen and ready to eat vegetables, were collected from a local supermarket, and about 3.03% of samples were positive for *L. monocytogenes* contamination, with 2.2% of raw vegetables, and 5. 6% of ready to eat vegetable contamination (Byrne et al., 2016).

Although Brazilian and New Zealand studies show different units to report the data, all the studies mentioned above were carried out either in the retail stores or farmer's markets, but no study was found on the post-packhouse scenarios and the persistence of *L. monocytogenes* (or any other bacterial pathogen), which refocussed the need to begin concentrating on the post-packhouse domestic supply chain scenario and *L. monocytogenes* prevalence in apples.

1.7 Review the effect of individual lineages of *L. monocytogenes* on fresh produce

As mentioned previously, *L. monocytogenes* is divided into four lineages; Lineage I, II, III, and IV. While doing the whole-genome analysis on three commercial tree-fruit packinghouses (apples) (P1, P2, P3), two out of three packinghouses had the highest incidence of lineage III, followed by lineage I and II, showing the possible connection of animal contamination in these facilities (Chen et al., 2022).

In another study of a microbial survey of ready-to-eat vegetables in China, it was found that 33.3% of *L. monocytogenes* isolates belong to lineage I, and 50% belonged to lineage II (Chen et al., 2019).

Cold storage studies have found serotype 1/2a (lineage II) to be more cold-tolerant than serotype 4b (lineage I) (Buncic et al., 2001; Hingston et al., 2017; Lianou et al., 2006). Food safety of tree-fruits, especially apples, is not as well researched as for vegetables and leafy greens (Chen et al., 2022). Although other fresh produce with high pH is a well-known to have a higher risk of contamination with *L. monocytogenes*, apples with low pH (< 4) are also regarded as a potential vehicle of *L. monocytogenes* (Chen et al., 2022), and postharvest practices like waxing might extend the survival of the pathogen for a long time (Macarisin et al., 2019). Currently, as there is no study available investigating individual lineages and their survival on apples, an in-depth understanding of the ecology, distribution and persistence of *L. monocytogenes* is essential.

1.8 Review the microscopic attachment of *L. monocytogenes* of apples during storage conditions

Harbourage sites, surface attachment, and biofilm formation are vital to the persistence of *L. monocytogenes* in the environment (Carpentier and Cerf, 2011). A biofilm is the union of cells made up of single or multiple species attached to each other or the surface through a self-made matrix called an extracellular polymeric substances (EPS) (Nowak et al., 2015), consisting of DNA, proteins and polysaccharides. The capacity to form biofilms in the harbourage sites enables *L. monocytogenes* to establish itself. These biofilms make the management of *L. monocytogenes* more difficult as the biofilm cells are more resistant to sanitisers and antibiotics (Giaouris et al., 2014; Van Houdt and Michiels, 2010). The presence of macrostructures of apples such as stem end, calyx and other shape abnormalities may provide more harbour sites for bacteria that are difficult to clean in the packhouse (Buchanan et al., 1999; Kenney et al., 2001).

The key steps of biofilm formation include adherence, where planktonic cells begin by adhering reversibly to a surface they are in contact by weak interactions. This is followed by establishing stronger interactions between themselves and the surface. These interactions are mediated by cellular appendages and by deposition of extracellular matrix (EM), resulting in the adherence becoming irreversible. These adherent cells multiply and produce microcolonies, eventually making a mature biofilm (Finn et al., 2023).

Previous literature studied the mechanism of *L. monocytogenes* attachment (Gorski et al., 2003; Reina et al., 2002) and internalisation (Chen et al., 2016; Macarisin et al., 2017) on the surface of fresh produce. Attachment of *L. monocytogenes* is affected by several factors such as temperature, bacteria features, produce surface properties, and exposure time (Reina et al., 2002). A few publications show the mechanism of *L. monocytogenes* attachment in various fresh produce, e.g. radish tissue (Gorski et al., 2003) and cucumber (Reina et al., 2002). (Gorski et al., 2003) showed that the attachment mechanism could also be dependent on the different temperatures and flagellar motility. Whereas Reina et al. (2002) showed that produce's surface properties, exposure time, bacterial features, and temperature contribute to the attachment. However, the attachment of *L. monocytogenes* to the surface of apples has not been widely studied (Pietrysiak and Ganjyal, 2018).

Apple morphology is divided into three parts; overall shape, macrostructures like stem and calyx region, and microstructure like lenticels and stomata (Pietrysiak and Ganjyal, 2018). Macrostructures, primarily stem and calyx end regions, may provide harbouring site for bacteria, which is difficult to clean (Kenney et al., 2001), even during high-pressure washing. Gala, Golden Delicious, and Granny Smith inoculated with *L. innocua* attached primarily to the stem and calyx end region of the apple peel. Apple's surface morphology is critical to understanding bacterial attachment (Pietrysiak and Ganjyal, 2018). However, no study of microscopic bacterial attachment of *L. monocytogenes* on apples i*n storage was* found.

1.9 Summary

Produce consumed fresh, does not undergo any lethal treatment (such as cooking) that kills all the pathogens before consumption. For this reason, if a pathogen enters the supply chain, it may be present during consumption (Abadias et al., 2006).

Reviewing studies on the effect of international and domestic supply chains on *L. monocytogenes* showed that all studies have only been carried out under static temperatures. It is not known whether *L. monocytogenes* survives or even grows under the dynamic temperature conditions that reflect real-world supply chains.

Storage and transportation are the two most important aspects of the apple supply chain, and there was inadequate information available on the post-packhouse persistence of *L. monocytogenes*. It is crucial to understand the effect of temperature on an international and domestic supply chain and how it affects the pathogen.

An in-depth study of *L. monocytogenes* lineages in the post-packhouse scenarios, it was found that although literature suggests that lineage II is predominantly found in the food and food processing environments, there has been a high prevalence of lineage I and III as well. For apples, due to the scarcity of information for individual lineages, an in-depth understanding of the ecology, distribution and persistence of *L. monocytogenes* lineages was required.

There is little information on *L. monocytogenes*' attachment on the apple surface during storage. Apple has various hidden areas like stem end and calyx, which can house various bacterial pathogens where sanitisers or other treatments cannot reach. Even if a small amount of *L. monocytogenes* entered the hidden structures, there could be a possibility of biofilm formation in the microstructures or growth of *L. monocytogenes*. Hence, attachment studies

of *L. monocytogenes* on various apple regions at different temperatures may provide important information.

This review outlined the importance of temperature in the apple supply chain and the persistence of *L. monocytogenes* in those conditions. Future storage studies that involve an in-depth real-world supply chain temperatures on real apple shipments, and then assess the survival of *L. monocytogenes* will have valuable applications for modelling the bacterial risks on apples through the supply chain. Also, an in-depth study on out of three lineages, which lineage of *L. monocytogenes* is most active in the apple supply chain, and their bacterial attachment using microscopy could be most valuable in understanding the bacteria.

1.10 Thesis aim and objective:

This research was aimed to examine the fate of different lineages of *L. monocytogenes* inoculums (in cocktail or individual strain scenarios) when temperature variations were investigated in the international and domestic fresh apple supply chains and how these pathogens attach on different regions of apple.

The main objectives were to

A. Monitor temperature variations of international and domestic apple supply chains
B. Determine the survival and inactivation kinetics of *L. monocytogenes* in open and closed calyx apple cultivars under the retrieved commercial temperatures from objective A, simulated in the laboratory
C. Determine the fate of different lineages of *L. monocytogenes* at high and low inoculum levels for commercial temperatures of apple cultivars, by investigating different testing methods.

D. Investigate the microscopic attachment of individual *L. monocytogenes* lineages on the apple cultivars at different inoculated regions when the apples were stored at different temperatures.

The thesis hypothesised that as the temperature in the apple supply chain is variable/dynamic in nature, the risk of *L. monocytogenes* population should increase under those conditions.

Preface to Chapter 2

Based on the export value, apples are one of the most important crops for New Zealand's economy. When apples are sent from New Zealand to other parts of the world, every exporter must follow the guidelines/criteria for the particular countries for *L. monocytogenes*. These guidelines show the acceptable levels of *L. monocytogenes* for that country. While the USA has a "zero tolerance" policy for *L. monocytogenes*, European countries like Germany, France, and the Netherlands have set a tolerance level of 100 colony-forming units (CFU) of *L. monocytogenes* per gram of food at consumption.

Although *L. monocytogenes* can enter at any step of a supply chain, there is little to no data available that quantifies the actual dynamic conditions when fruit is sent from New Zealand to other parts of the world. Hence, this challenge study becomes crucial to assess if dynamic temperatures have any effect on *L. monocytogenes* survival.

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Decline of *Listeria monocytogenes* on fresh apples during long-term, low-temperature simulated international sea-freight transport

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Chapter 2: The effect of dynamic temperatures on *Listeria monocytogenes* in the international apple supply chain

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Abstract

Listeria monocytogenes has caused outbreaks of foodborne illness from apples in the USA and is also a significant issue for regulatory compliance worldwide. Due to apple's significance as an important export product from New Zealand, we aimed to determine the effect of long-term, low-temperature sea-freight from New Zealand to the USA and Europe, two key New Zealand markets, on the survival and/or growth of *L. monocytogenes* on fresh apples. Temperature and humidity values were recorded during a shipment to each market (USA and Europe), and then the observed variations around the 0.5°C target temperature were simulated in laboratory trials using open ('Scired') and closed ('Royal Gala' for the USA and 'Cripps Pink' for Europe) calyx cultivars of apples inoculated with a cocktail of 10^7-10^8 cells of seven strains of *L. monocytogenes*. Samples were analysed for *L. monocytogenes* quantification at various intervals during the simulation and on each

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occasion, an extra set was analysed after a subsequent 8 days at 20°C. When both the sea-freight simulations concluded, *L. monocytogenes* showed 5 log reductions on the equatorial surface of the skin of apples but only about 2.5 log reductions for the USA and about 3.3 log reductions for Europe in the calyx. Cultivar type had no significant effect on the survival of *L. monocytogenes* for both sea-freight simulations, either in the calyx or on the skin ($P > 0.05$). Most of the reduction in the culturable cells on the skin occurred during the initial 2 weeks of the long-term storage simulations. There was also no significant difference in the reduction of *L. monocytogenes* at 0.5 or 20°C. No correlation was observed between firmness or total soluble solids and the survival of *L. monocytogenes*. Because the inoculated bacterial log reduction was lower in the calyx than on the skin, it is speculated that the risk of causing illness is higher if contaminated apple cores are eaten. The result suggested that international sea-freight transportation does not result in the growth of *L. monocytogenes* irrespective of time and temperature. The results of this study provide valuable insights into the survival of *L. monocytogenes* on different apple cultivars that can be used to develop effective risk mitigation strategies for fresh apples during long-term, low-temperature international sea-freight transportation.

Keywords: open and closed calyx apples, refrigerated storage, risk analysis, transport, room temperature storage, inactivation.

2.1 Introduction

Listeria monocytogenes is a foodborne bacterial pathogen that is ubiquitously found in soil, manure, water, and the packhouse environment (Marik et al., 2020; Tabit, 2018). It can be transferred to fresh produce in the field or postharvest with several reported cases found on ready-to-eat tree fruits such as stone fruit and apples (Angelo et al., 2017; Buchanan et al., 2017; Jackson et al., 2015; Marik et al., 2020). *L. monocytogenes* is classified as a Gram-positive psychrotrophic organism, enabling it to grow at variable temperatures, from –1.5°C to 45°C. It also has a tolerance for high salt concentrations and variable Ph ranging from 4.0–9.6 (Santos et al., 2019). Ingestion of foods contaminated with *L. monocytogenes* may lead to listeriosis, especially in immunocompromised individuals and pregnant women (Zhu et al., 2017). Compared with other diseases caused by foodborne pathogens, listeriosis is relatively rare but has a high fatality rate of up to 30% (Scallan et al., 2011). As well as immunocompromised people, *L. monocytogenes* can cause illness in a healthy population when the consumed food has high concentrations of the bacteria (1.9×10^5 CFU/g to 1.2×10^9 CFU/g), although it depends on the types of serovars (FSANZ, 2013). While the USA has a zero tolerance of *L. monocytogenes* in ready-to-eat foods (RTE), several European countries such as Germany and France have set tolerance levels of 100 colony forming units (CFU) of *L. monocytogenes* per gram of food consumed or not (Tabit, 2018). Food Standards Australia and New Zealand (FSANZ) set up two sets of criteria for RTE products, based on whether the growth of *L. monocytogenes* will occur (should be absent in 25 g) or the growth of *L. monocytogenes* will not occur (should be less than 100 CFU/g) (FSANZ, 2014). Although different between countries, these guidelines have become a global reference point for consumers, food producers, processors, and the international food trade. Tree fruit, such as apples, are considered to be RTE food and are routinely tested for the prevalence of

bacteria as part of risk management strategies associated with food safety certifications (Luber, 2011; Tabit, 2018).

Despite these testing and monitoring, an outbreak of listeriosis occurred from the consumption of caramelised apples in 2014-2015 in the USA. The outbreak resulted in 35 cases with 20% mortality (Angelo et al., 2017; Buchanan et al., 2017; CDC, 2015). The federal government's epidemiological investigations identified the source of contamination to a single caramel apple grower in California. A sampling of the packhouses showed polishing brushes, drying brushes, a packing line drain, a wooden bin, and an automatic packing line also tested positive for *L. monocytogenes,* and it is believed that the contamination was exacerbated when sticks were inserted into the fruit as part of the caramel apple production process (Beecher, 2016). Although most cases were linked to the consumption of caramel apples, there were three other cases in which the food vehicle was possibly either fresh cut or whole apple (CDC, 2015; Macarisin et al., 2019; Salazar et al., 2016b). This outbreak was the first ever *L. monocytogenes* outbreak associated with apples and highlighted that fresh apples could be a severe risk for *L. monocytogenes* contamination (Angelo et al., 2017). There have been a few outbreaks recently as well. E.g. In 2017, an apple company in Michigan recalled 'Gala', 'Fuji', 'Honeycrisp', and 'Golden Delicious' apples due to the presence of *L. monocytogenes* (FDA, 2020). In the same year, another company based in Michigan recalled sliced apples due to the presence of *L. monocytogenes* (FDA, 2017). In 2019 in Michigan, 'McIntosh', 'Jonathan', 'Honeycrisp', 'Fuji' were recalled due to the presence of *L. monocytogenes* (FDA, 2019). These outbreaks also revealed the lack of information on the behaviour of *L. monocytogenes* on fresh apples during storage, especially long-term and at low temperatures, post-packhouse.

After packing, the postharvest life of apples is significantly longer than other tree fruits, such as avocadoes and citrus, often reaching the consumer as long as six or more months after

harvest. During this time, apples are exposed to various microbial contaminants from the air, packhouse equipment, vehicles, and food handlers (Macarisin et al., 2019). Thus, it is essential to evaluate the capacity of *L. monocytogenes* to grow on apples during storage and potentially cause illness (Macarisin et al., 2019).

The survival and growth kinetics of *L. monocytogenes* on the surface of apples during postharvest storage and transport is not well researched. In the current literature, there are few studies on how the survival of *Listeria* may vary in the actual supply chain, where the temperature is under dynamic rather than static conditions. Furthermore, the morphological differences among apple cultivars (open vs closed calyx, comparatively) is a factor that might affect pathogen survival on the apple surface by offering a hidden environment that promotes its growth (Macarisin et al., 2019). To the best of the authors' knowledge, there is no information regarding the survival of *L. monocytogenes* among apple cultivars (open vs closed calyx) during long-term, low-temperature, simulated international sea-freight transport, such as typically happens for apple exports from New Zealand.

New Zealand has an agriculture-based economy in which apples are exported to 65 countries and are crucial for New Zealand trade, accounting for almost a quarter of the export value of fresh produce (MBIE, 2017). Maturity parameters such as starch pattern index (SPI), soluble solids, and firmness are the key to determining the storage potential for apples (Barber, 2019). We found no evidence in the literature on the relationship between apple maturity and *L. monocytogenes* persistence, which could have implications for the New Zealand apple industry. Even though New Zealand has recorded no outbreak of listeriosis or had a recall regarding apples, the potential exists for *L. monocytogenes* to be present on apples, necessitating a rigorous risk assessment of the food safety risk under New Zealand export conditions, especially at low temperatures such as 0.5°C which is regarded as a standard in the apple industry and often used while sea-freighting.

A USA study during postharvest storage suggested that cold temperature is insufficient to eliminate *L. monocytogenes* on fresh apples (Sheng et al., 2017). However, this storage study was performed under static temperatures in a lab. There have been other studies as well that were done under static temperatures (Macarisin et al., 2019; Salazar et al., 2016b). It is unknown whether *L. monocytogenes* survives or even grows under the dynamic temperature conditions that reflect reality during domestic or international supply chain transport. Also, because the postharvest packing of apples does not usually have any "kill step" that can potentially inactivate *L. monocytogenes* (Pietrysiak et al., 2019), understanding the inactivation behaviour of *L. monocytogenes* during dynamic temperatures is essential. Under stress, microorganisms do not decline linearly but in a non-linear fashion. The non-linear survival curve of bacteria has been described in models such as those of Gompertz (Kahraman et al., 2017), and Weibull and Baranyi (Huang, 2014) models. The Weibull model is the most useful as it can describe various survival patterns over time (Buzrul et al., 2005). Therefore, the objective of this study was to determine the effect of long-term, low-temperature sea-freight from New Zealand on the survival and/or growth of *L. monocytogenes* by: (i) recording and comparing actual temperature and humidity during long-term, low-temperature shipments to two key New Zealand markets, the USA and Europe; (ii) determining *L. monocytogenes* survival in open and closed-calyx cultivars on two regions of apples (skin and calyx) during international sea-freight simulations; (iii) assessing the response of *L. monocytogenes* to ambient shelf-life temperature (household storage conditions) after long-term, low-temperature storage; (iv) exploring the inactivation kinetics of *L. monocytogenes* for each cultivar at each inoculation region; and (v) investigating whether destructive measurements such as total soluble solids and firmness could be correlated with survival of *L. monocytogenes*.

2.2 Materials and methods

2.2.1 Export transport conditions

To simulate export shipping conditions for subsequent *L. monocytogenes* challenge trials, temperature profile data from a refrigerated shipping container bound for the USA were recorded. To make the least disruption possible for the other carton and then the whole pallet, temperature loggers were placed on the surface of the apples in a single carton. Temperature loggers were iButton®, Tinytag view, LogTag, Ebro, Xsense, Temprecord. The carton with temperature loggers was placed on the outside edge of the top row of a shipping pallet to expose the fruit to more significant temperature changes than the centre of the pallet. The carton was then sea-freighted as part of a commercial consignment of 210 pallets to the USA. After sea-freight, the loggers were recovered on arrival in the USA and returned to Auckland, New Zealand, for data recovery. A similar exercise was performed for a shipping consignment to Europe. For both markets, the temperature was monitored on an hourly basis. The challenge studies examined the survival behaviour of *L. monocytogenes* during simulated long-term, low-temperature, international sea-freight transport over an extended storage time of 12 weeks for the USA and 20 weeks for Europe.

2.2.2 Challenge trial fruit source

For the USA transport simulation, commercially graded export quality un-waxed apples (*Malus* x *domestica*) 'Royal Gala' and 'Scired' were selected as they represent the closed and open calyx cultivars, respectively. Exporting waxed or unwaxed apples usually depended on the demand and requirements of the particular country. For the Europe transport simulation, 'Cripps Pink' and 'Scired' were chosen as closed and open calyx cultivars, respectively. Apples of 100-count size per carton with no bruising, cuts or scars were obtained from a commercial packhouse.

2.2.3 Selection of L. monocytogenes strains

To account for variation in growth and survival among strains, six genetically different *L. monocytogenes* isolates from horticultural sources (PFR46G06, PFR46E10, PFR40I07, PFR41I04, PFR41F08, PFR41H05), and one clinical strain (Scott A) were obtained from The New Zealand Institute for Plant and Food Research Limited's (PFR) culture collection and used to create a seven-strain bacterial cocktail (Table 2.1). Because there are few types of serotypes representing every lineage, the sequence types are mentioned in Table 2.1. Before the experiment, the isolates were subjected to whole genome sequencing for taxonomic analysis. The seven isolates were grown in tryptic soy-yeast extract broth at 37°C. Genomic DNA was extracted using a bacterial genomic DNA extraction kit (Geneaid Presto^{TM} Mini gDNA bacterial kit, Geneaid Biotech Ltd, New Taipei City, Taiwan) according to the manufacturer's protocol. The DNA was sent for Whole genome sequencing, performed by Westmead Institute for Medical Research & Centre for Infectious disease and microbiology, Sydney, Australia. The lineages and distribution of sequence types identified from each strain are provided in Table 2.1.

Table 2.1: *Listeria monocytogenes* strains obtained from Plant and Food Research (PFR) culture collection, including the lineage and sequence type (ST) determined by whole genome sequencing*

L. monocytogenes strains	Lineage	ST
PFR46G06	1	4
PFR46E10	1	1262
PFR40I07	2	155
PFR41I04	2	155
PFR41F08	3	299
PFR41H05	3	299
Scott A[1]	1	290

*as described by Briers et al. (2011).

2.2.4 Inoculum preparation

L. monocytogenes individual strain purity was confirmed by streaking onto tryptic soy agar with 0.6% added yeast extract (TSAYE) (BD, Becton, Dickinson & Company, USA) and then onto the selective media *Listeria* CHROMagar™ plates (CHROMagar™, Paris 75006, France) and checking blue colony appearance and uniformity. Pure cultures were then grown in tryptic soy broth with 0.6% added yeast extract (TSBYE) (BD, Becton, Dickinson & Company, USA) for 48h at 37°C to achieve a stationary growth phase. Strains were then pelletised by centrifugation at 4000 rpm (3220g) (Gyrozen model 1736R, Seoul, South Korea) for 10 min at 4°C. The resulting pellets were washed twice and re-centrifuged with 0.1% peptone (Bacto™, BD Biosciences, USA). An equal amount of each *L. monocytogenes* strain was combined to make a seven-strain cocktail with a final concentration of 10^8 colony forming units (CFU/mL) in 0.1% peptone that was subsequently used for apple inoculation.

2.2.5 Inoculation of apples

In both of the transport simulations, whole unwaxed apples were transferred from cold storage (0.5°C) before inoculation and held at room temperature (~20°C) overnight to equilibrate apple temperature. There are quite a few ways by which apples could conceivably become contaminated. It could be by splash of contaminated water, by coming in contact with contaminated surfaces such as packhouse contact surfaces or by being immersed in contaminated water such as flumes used for moving apples in a packhouse. We chose spot-inoculation to simulate the splash of contaminated water. The apples were spot-inoculated with the *L. monocytogenes* cocktail as follows. For each cultivar, apples were divided into two groups: (i) calyx inoculation, where one 50 µL aliquot of *L. monocytogenes* cocktail was pipetted into the calyx of the apple; and (ii) skin inoculation, where two drops of 25 µL each were pipetted on the equatorial region (Fig. 2.1). All apples were air-dried for 3 h in a biosafety class II cabinet at room temperature until visibly dry.

A B

Figure 2.1: *Listeria monocytogenes* inoculation in the calyx (A) and on the skin surface (B) of an apple.

2.2.6 Transport simulation of apples and sampling

Inoculated apples were placed in poly-lined cardboard boxes and stored in a temperature-controlled room to simulate previously derived temperature profiles (Figures 2.2A and 2.2B). To simulate the temperatures, A -30°C freezer/cold store room was modified to become a

cool room capable of operating over a range of programmed temperatures from -5°C to +30°C. The 8 KW split refrigeration system was controlled by the addition of a Yudian AI-518 artificial intelligence proportional integral derivative (PID) temperature controller (Yudian Automation Technology Co., Ltd., Hong Kong) and a proprietary control programme prepared using LabVIEW software (v2019, National Instruments Inc., USA) was used to control the temperature profile. The temperature dead-band range was set to 0.8°C and the auxiliary output high deviation level set to 0.9°C. The auxiliary output is used to control the compressor. During the heating cycle power to the evaporator defrost heaters was controlled with a solid-state relay using pulse width modulation. The PID temperature controller parameters and heater power level are set by the LabVIEW programme dependent on the temperature level required. The LabVIEW program tracked the temperature profile and transmitted the current temperature set point to the temperature controller every minute.

As with commercial shipments, trays of fruit were placed in polystyrene liners inside the boxes to maintain humidity and prevent water-loss. The boxes were stored in air (not a controlled atmosphere) but it is assumed that fruit respiration will have altered the atmosphere composition similarly in both the commercial and simulated shipments. The temperature and humidity were monitored at 1 h intervals using Cydiance real-time temperature and humidity logger (Cydiance, Shanghai, China), and also monitored at 30 min intervals using Maxim type-L iButton temperature loggers (Maxim Integrated, USA). Fruit sampling to quantify *L. monocytogenes* was conducted on days 0 and 1, 2, 4, 8, and 12 weeks for the USA trial and days 0 and 1, 2, 4, 8, 12, 16 and 20 weeks for the Europe trial.

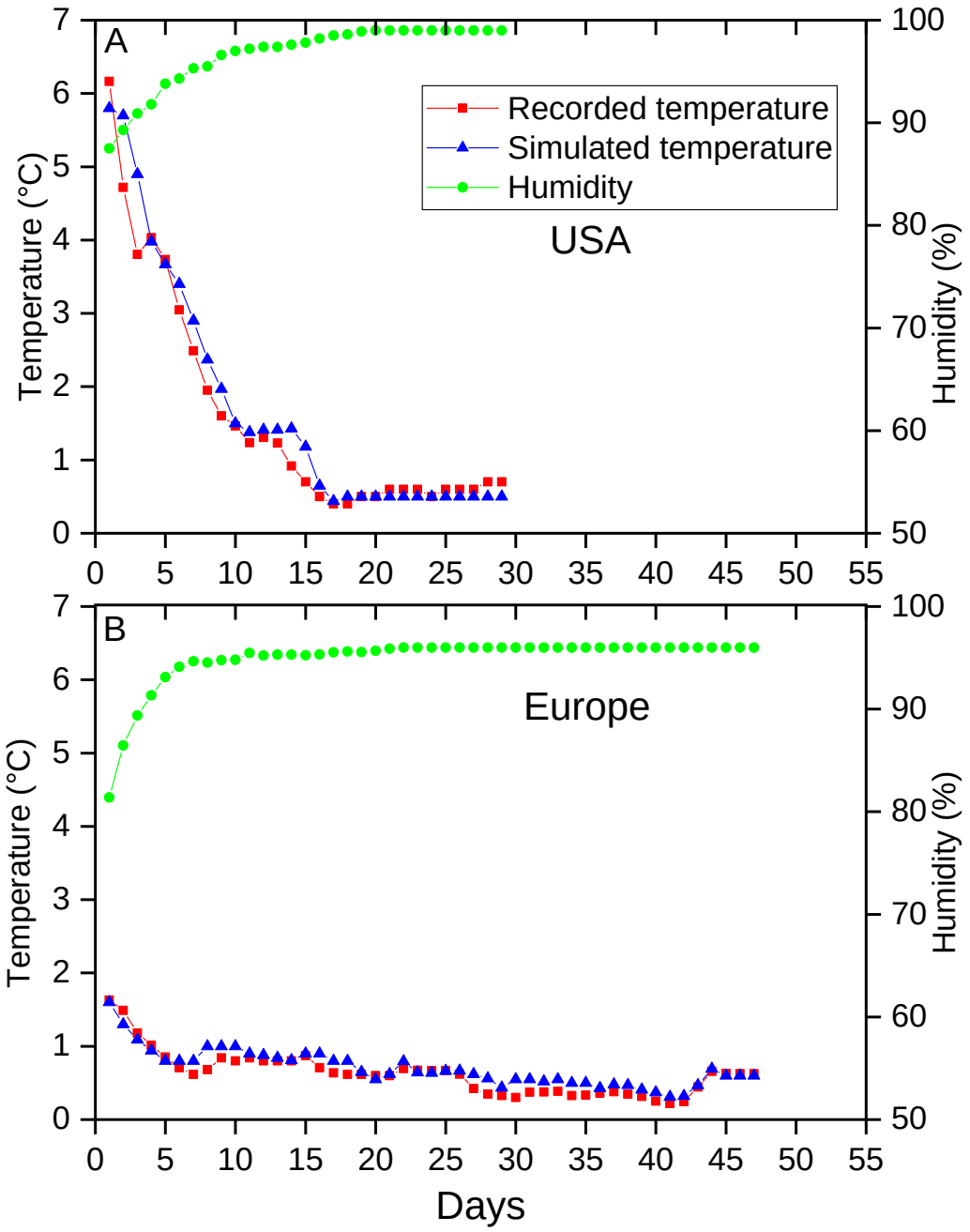

Figure 2.2: Recorded and simulated temperature profile of apples during sea-freight from New Zealand to the USA (A) and Europe (B)

At each refrigerated storage time-point, nine fruit were sampled for each cultivar per inoculation and *L. monocytogenes* populations were determined on the inoculated apples. At each refrigerated storage time-point, a further nine apples for each cultivar per inoculation were randomly sampled and stored for 8 days at 20°C, to simulate a household shelf-life time and temperature, before being similarly tested. Generally, in postharvest studies, the ambient shelf life temperature is studied at 20°C, and the shelf life duration (the estimate amount of time to consume apples in any household) is recorded at 7 – 8 days.

2.2.7 Microbial enumeration of apples

Three sets of three apples of each inoculation method were quantitatively assessed for *L. monocytogenes*, sampling either the skin or the calyx using the most probable number (MPN) method to detect stressed organisms (Osborne and Bremer, 2002). For skin inoculations, each sample, i.e. three apples, were placed into a sterile bag with 400 mL of Buffered *Listeria* Enrichment Broth (BLEB) (Acumedia, Lansing, Michigan, USA) and hand massaged for 2 min. Triplicate 2 mL aliquots of BLEB wash solution from each bag were transferred into 15 mL falcon tubes. Triplicate 200 µL BLEB aliquots were dispensed in microtiter well plates, followed by 10-fold serial dilutions. Aliquots and the original bag were then incubated for 48 h at 30°C for enrichment. After 48 h, 2 µL aliquots of the enriched BLEB from each of the bags, tubes and microtiter wells were plated onto pre-gridded selective CHROMagar™ *Listeria* plates, which were then incubated for 48 h at 37°C for detection of the inoculated *L. monocytogenes*. Blue colonies were recorded as positive readings for *L. monocytogenes*.

For the calyx inoculations, the core of the apple was removed with a sterile cork-borer (16-mm diameter), weighed, and homogenised in a laboratory stomacher (Smasher, AES Chemunex, AES Laboratory, France) for 2 min in BLEB (1:10). This was followed by the same enumeration protocol used for the skin inoculations described above.

MPN values were calculated using the Bacteriological Analytical Manual spreadsheet (Blodgett, 2010). All results were expressed as $\log_{10}$ MPN per apple with a detection limit of 1.37 $\log_{10}$ MPN per apple from the tubes and wells and presence/absence in three apples from the bags.

2.2.8 Inactivation Kinetics

The inactivation kinetics of *L. monocytogenes* during long-term, low-temperature simulation of apples were evaluated based on the Weibull model. The Weibull model assumes that all the microorganisms rarely die at the same time when exposed to an agent; hence a survival curve is a cumulative distribution of lethal effects and follow non-linear kinetics (van Boekel, 2002). Experimental data were fitted in decimal logarithmic form as follows:

$$\log N_t/N_0 = -b * t^{\beta} \quad \text{Eq. (1)}$$

Where N (t) is the number of microorganisms surviving after the storage simulation time t and N_0 is the initial number of microorganisms. The b parameter ($min^{-\beta}$) is defined as:

$$b = 1/ 2.303 * (1/ \alpha)^{\beta} \quad \text{Eq. (2)}$$

Where α (day^{-1}) is the coefficient in the Weibull distribution, known as the scale parameter, and β is the shape parameter. If $\beta < 1$, then it shows that the remaining cells can adapt to the stress, i.e. the remaining cells are resistant to change (also known as a tailing effect). If $\beta > 1$, the remaining cells become damaged because of the treatment and possibly cannot revive themselves (also known as a shoulder effect). The model describes first-order kinetics in rare cases when $\beta = 1$ (Bozkurt et al., 2016; Buzrul et al., 2005; van Boekel, 2002).

2.2.9 Physical assessment of apples

At each storage time-point, another subsample of five fruit was assessed for the quality characteristics of firmness, soluble solids concentration (SSC), and starch pattern index (SPI) using destructive postharvest measurements.

Fruit firmness was assessed using a fruit texture analyser (Guss, Model GS14, South Africa) fitted with 11.1 mm Effigi^{TM} penetrometer probe (Nock and Watkins, 2013). Two measurements were made per fruit on paired surfaces on the opposite side of the fruit and averaged.

Starch is a key parameter in determining actual ripeness and storage potential of apples. The test provides an indication of the amount of starch (amylose) which has converted to sugar and as such advanced ripening, which also corresponds with increasing ethylene status. Starch accumulates in apples during the growing season and is hydrolysed to sugar in the latter stages of maturation and development. The SPI was determined after cutting the fruit equatorially and spraying the cut surface of each fruit with an iodine solution (2.5 g iodine and 10 g/L potassium iodide in distilled water). The degree of blue/black staining was assessed after 60 s and rated according to the 7-point scale chart of apple maturity from ENZAFRUIT New Zealand International, where 0 is completely stained (high starch), and 6 is no surface staining (low starch) (Johnston et al., 2009).

The SSC (°Brix) was determined with an Atago digital refractometer (Atago, model PAL-1, Japan) using juice expressed during fruit firmness measurement.

2.2.10 Statistical analysis

Microbial and destructive data were analysed using GenStat (version 18th; VSN International Ltd). Long-term low-temperature simulation data were analysed using a two-way analysis of variance (ANOVA) followed by post-hoc Tukey's test. Results are presented as means with

standard deviations in the parenthesis in the text. Log change/per day data of ambient shelf life was compared with long-term low temperature data using a t-test. Pearson's correlation was carried out to see the relationship between log MPN/apple and firmness with $P < 0.05$ considered statistically significant. All the figures were prepared using the Origin software 2017 (OriginLab Corporation, Northampton, USA). For inactivation kinetics, statistical and non-linear regression analyses were performed using the SPSS statistical package version 26 (IBM Corporation, Armonk, NY, USA).

2.3 Results and Discussion

Because the temperatures retrieved were from the commercial sea-freight bound to the USA and Europe, the temperatures were considered representative of a typical sea-freight journey. Figures 2.2A and 2.2B represent the temperatures of the recorded and simulated sea-freight shipments and recorded humidity for the USA and Europe. Temperature for the sea-freight shipments to the USA dropped from 6°C (0.12) to 0.5°C (0.22) in the first 2 weeks of transport and then remained constant (~0.5°C) (Figure 2.2A). The recorded humidity around the apples increased from 87% to 99% over the first 20 days and remained constant. In contrast, for the Europe shipment, temperature changed from 2°C to 0.7°C in 1 week and remained between 0.7 - 0.5°C ± 0.2°C for the rest of the shipment (Figure 2.2B). Humidity increased from 81% to 94% over the first 6 days and only gradually increased to 96%. In both experiments, high humidity and low temperatures did not promote condensation. Humidity is generally high in the shipping containers. High humidity reduces the weight loss in apples. Also, low humidity could lead to apples undergoing stress, which could result in increased ethylene production, and ultimately apples getting rotten (Cargo Handbook, 2023).

The initial titres of *L. monocytogenes* inoculum applied to the apples were 10^8 CFU/ml for the USA and Europe simulations. Four hours after inoculating apple samples, the mean recovered titres for USA simulations were 7.69 and 7.25 $\log_{10}$ MPN/apple for 'Scired' and 'Royal Gala'

calyces, respectively, respectively 7.42 $\log_{10}$ MPN/apple for both 'Scired' and 'Royal Gala' skin inoculations. For the Europe simulation, the recovered titres were 7.02 and 6.99 $\log_{10}$ MPN/apple for 'Scired' and 'Cripps Pink' calyces, respectively, and 7.18 $\log_{10}$ MPN/apple for both 'Scired' and 'Cripps Pink' skin inoculations. The slight drop in *L. monocytogenes* titre after inoculation could be due to organisms dying during the drying process, or inability of the hand massage method to remove all organisms from the apple surfaces, or differences in the accuracy of the two methods (plate counts compared with MPN). The former is considered most likely as hand massage would be expected to give better recovery from skin inoculations than calyx. In addition, because of the ability of the enrichment step in BLEB to recover stressed and injured cells, the MPN method can typically be expected to have higher counts than a plating method applied to the same sample.

Figures 2.3 and 2.4 showed that *L. monocytogenes* survived during 12- and 20-weeks' simulated sea freight storage, albeit at lower concentrations after the completion of simulation. Final *L. monocytogenes* concentrations were 5.34 and 4.67 $\log_{10}$ MPN/apple for 'Scired' and 'Royal Gala' for the USA simulations in the calyx respectively, a log reduction of 2.35 and 2.58 respectively. For both the cultivars for skin, final concentration was 2.19 $\log_{10}$ MPN/apple, log reduction of 5.24 in comparison to the initial values (Figure 2.3A), respectively. For Europe simulations, the final concentrations were 3.78 and 3.62 $\log_{10}$ MPN/apple for 'Scired' and 'Cripps Pink' in the calyx, respectively, a log reduction of 3.24 and 3.37 respectively (Figure 2.4A).

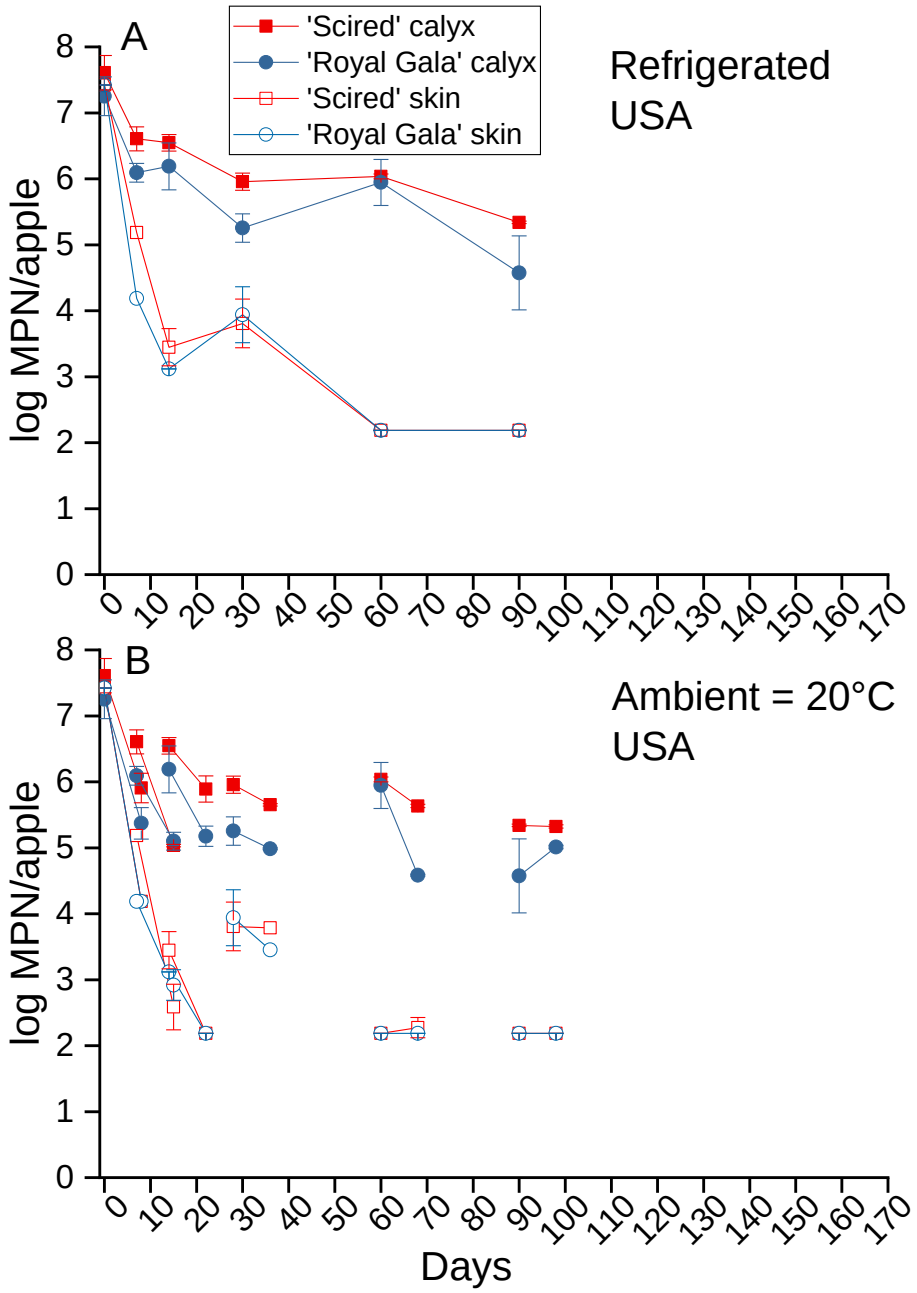

Figure 2.3: Survival of inoculated *Listeria monocytogenes* on the skin and calyx of apples during laboratory-simulated storage for commercial refrigerated shipment with a final temperature at ~0.5°C for 12 weeks for the USA (A), followed by ambient shelf-life storage at 20°C for 8 days (B). Figure 2.3(B) data are shown as a two-point segment graph, where one point is connected to the other point (log values in this case) by a line to show the difference between the two log values. In this case, one point is shown as a log value of the bacteria at the start of ambient shelf-life temperature and the other point is after 8 days of

shelf life at ambient temperature. These two points are connected by a line to show how much the bacteria have survived at the end of shelf life compared with at the beginning. Error bars are represented as standard deviations.

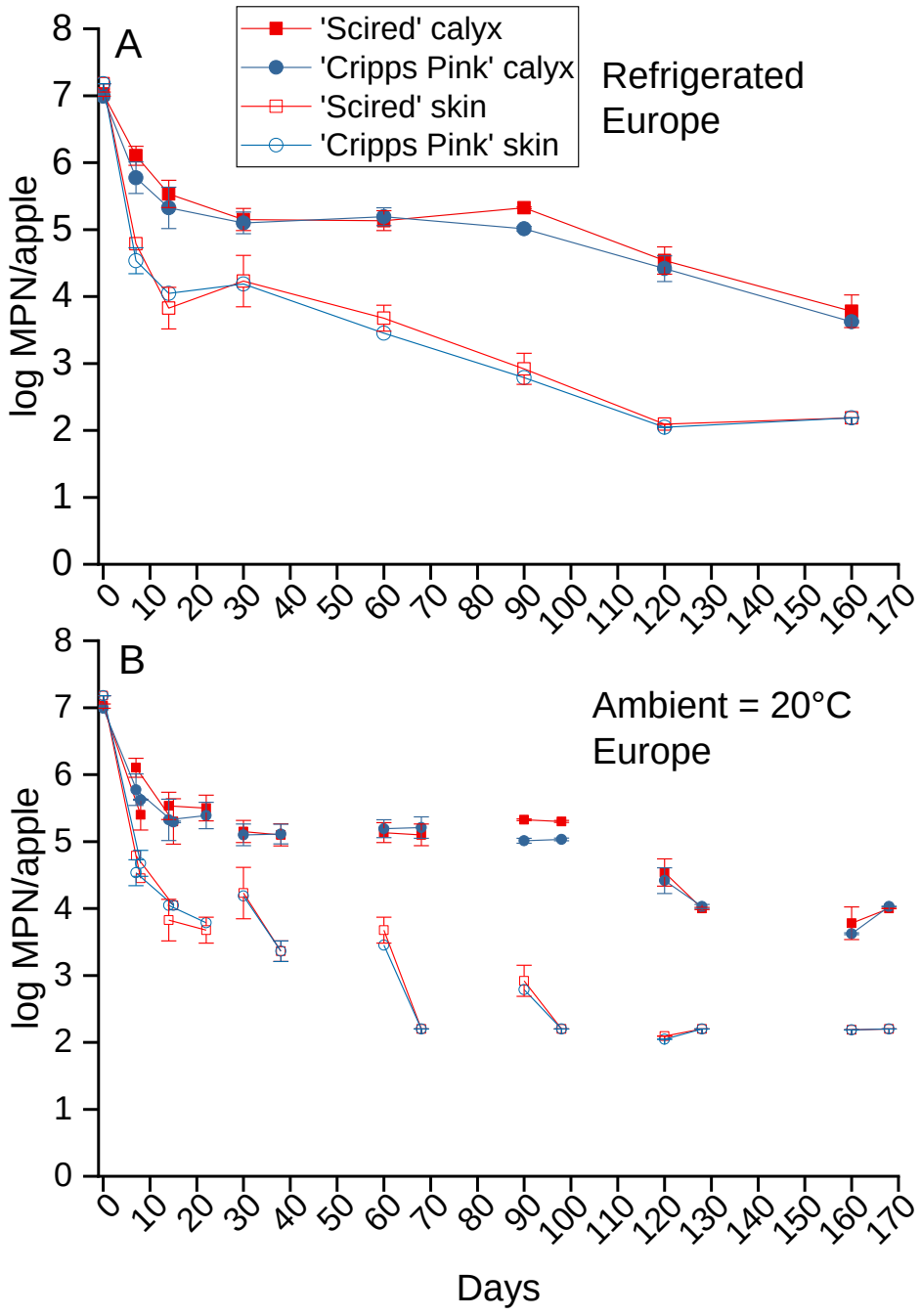

Figure 2.4: Survival of inoculated *Listeria monocytogenes* on the skin and calyx of apples during laboratory-simulated storage for commercial shipment with final temperature at

~0.5°C, for 20 weeks for Europe (A), followed by ambient shelf-life storage at 20°C for 8 days (B). Figure 2.4(B) data are shown as a two-point segment graph, where one point is connected to the other point (log values in this case) by a line to show the difference between the two log values. In this case, one point is shown as a log value of the bacteria at the start of ambient shelf-life temperature and other point is after 8 days of shelf life at ambient temperature. These two points are connected to each other by a line to show how much the bacteria have survived at the end of shelf life compared with at the beginning. Error bars are represented as standard deviations.

For both the cultivars for skin, final concentration was 2.18 $\log_{10}$ MPN/apple, log reduction of 5 in comparison with the initial inoculum (10^8 CFU/ml). The persistence of *L. monocytogenes* for two cultivars ('Royal Gala' and 'Scired') for the USA and ('Scired' and 'Cripps Pink') for Europe, at two different inoculation regions (skin and calyx), shows a rapid log reduction in *L. monocytogenes* populations occurred within the first 2 weeks of simulated transport. For the USA, this was a log reduction of 1.06 and 1.14 $\log_{10}$ MPN/apple for 'Royal Gala' and 'Scired', respectively, in the calyx, and 4.30 and 3.98 $\log_{10}$ MPN/apple on the skin, respectively (Figure 2.3A). In contrast, for Europe, the log reductions in the first 2 weeks were 1.49 and 1.67 $\log_{10}$ MPN/apple in the calyx for 'Scired' and 'Cripps Pink', respectively, and 3.35 and 3.13 $\log_{10}$ MPN/apple on the skin, respectively. After 2 weeks, *L. monocytogenes* populations declined slowly or remained constant for the skin and calyx. (Figure 2.4A). Log values of surviving *L. monocytogenes* were significantly ($P < 0.05$) higher in the calyx than on the skin of apple cultivars. This result agreed with the previous work done by Macarisin et al. (2019), which also suggested that bacteria survived better in the calyx.

Apple's surface is intact. Bacteria grow poorly on those places (like trichomes and lenticels) where there is low potential for the microorganism to penetrate intact barriers, e.g. on the surface of the apple. Growth on the intact surfaces is not common as bacteria do not possess any enzymes to break the skin of the produce (MSU, 2001). In the current study, *L. monocytogenes* on the skin of the apple were in a stressful environment for survival. No nutrients or protection were available on the barren apple surface, and they were already

stressed from the transfer from nutrient media into 0.1% peptone solution and subsequent drying on apple surfaces. Based on these conditions, we hypothesised that they died quicker in first 2 weeks post inoculation. In the calyx, the bacteria may be protected and hidden in microstructures such as cracks and crevices of the apple. These may serve as a harbour site for bacterial attachment by physical entrapment (Pietrysiak and Ganjyal, 2018), e.g. bacterial attachment of *Salmonella* Chester in the apple calyx and stem were much higher (94%) than on the skin area (6%) (Liao and Sapers, 2000). Also, generally, Gram-positive bacteria are more particular in their nutritional requirement and thus are not able to synthesise certain nutrients required for growth (Jay, 2000). Thus, although the calyx appeared to provide a more protected environment, it did not appear to provide the accessible nutrients necessary for growth. It is important to mention that the fruit chosen was of export quality, however bruises, punctures, small abrasions may change the findings, but it needs further study.

The morphology of the fruit is also crucial for bacterial survival in the supply chain. Some positions on the apple, particularly the stem and calyx, are challenging to clean in the packhouse, which means they can provide shelter for bacteria (Buchanan et al., 1999; Pietrysiak and Ganjyal, 2018). Also, microstructures such as lenticels and trichrome on apple skins may protect the bacteria from cleaning agents due to surface tension (Pietrysiak et al., 2019).

Because the core of the apple was taken out and was homogenised for sampling the calyx, acidic apple flesh will have been included (see Materials and Methods). Higher recovery from the calyx samples compared to the skin suggests that *L. monocytogenes* may have had an acid tolerance response, in which *L. monocytogenes* can use several mechanisms (such as the glutamate decarboxylase (GAD) system) to maintain its internal pH (Bucur et al., 2018). This adaptation towards the acid environment not only enhances the survival of the pathogen,

but also protects the bacteria against other adverse environmental conditions (Ryan et al., 2008).

Another explanation for higher log reduction on the skin of the apple compared with the calyx could be related to the presence of viable-but-non-culturable cells (VBNC). Conventional enrichment techniques cannot detect VBNC cells and hence they pose a real problem to the food industry. Many foodborne pathogens such as *L. monocytogenes* enter a VBNC state because of environmental stress such as lack of nutrients or unfavourable temperature. In the current study, because *L. monocytogenes* inoculated in the calyx is more sheltered compared with the skin, there is a chance that *L. monocytogenes* on the skin might have gone into a VBNC state (Highmore et al., 2018).

To assess whether any food product supports the growth of *L. monocytogenes*, it is important to know whether the background microbial population inhibits, allows or enhances the growth of *L. monocytogenes* (Zilelidou and Skandamis, 2018), e.g. the absence of natural microbial load in hot-smoked fish products leads to the rapid growth of *L. monocytogenes* (Zilelidou and Skandamis, 2018). Growth of *L. monocytogenes* cannot be considered only as a result of food's physicochemical parameters but also the combination of various microbial communities (Powell, 2004). The initial decline in *L. monocytogenes* counts in the first 2 weeks of cold storage could be linked to competition between *L. monocytogenes* and other microorganisms that limit its survival and proliferation. For example, (Carvalheira et al., 2010) identified *L. innocua* as an antagonist of *L. monocytogenes*, resulting in suppressed growth. Yeast epiphytes, can also affect the survival of *L. monocytogenes* (Macarisin et al., 2019) and the presence of lactic acid bacteria (LAB) showed anti-microbial activity against *L. monocytogenes* (Oliveira et al., 2012).

The ambient shelf-life temperature simulations (20°C) graphs for the USA (Figure 2.3B) and Europe (Figure 2.4B) showed log values are significantly different ($P < 0.05$) in the calyx and the skin, a similar pattern observed in the cool-stored simulated temperatures (Figure 2.3A and 2.4A). Similar to the cool-stored temperature simulations for both USA and Europe, there was a substantial log reduction in the first 2 weeks. The initial drop in the ambient shelf-life temperature simulation at 20°C can be described by the same explanation given in the cool store temperature simulation above.

Table 2.2 and 2.3 present the log change per day between the cool stored simulation and ambient shelf-life simulation. The t-test was used to compare bacterial log change per day of two cultivars at the two inoculation regions at both export market cool storage simulations and the ambient shelf-life simulations. t-Test results suggested that for both the USA (Table 2.2) and Europe (Table 2.3) simulations, there was no difference in the bacterial log change per day between the cool stored and ambient shelf-life simulations either in the calyx or on the skin ($P > 0.05$) (Table 2.2 and 2.3). The result suggested that under the dynamic and ambient conditions selected in this study, storage temperature is not functioning as an intervention for bacterial persistence. In particular, the decline in survivors over the first two weeks is independent of storage temperature.

Table 2.2: Log change/day comparison of inoculated *Listeria monocytogenes* on apples during simulated cool store and ambient shelf life (for eight days) over 12 weeks for the USA, and t-test results between the log change/day values of cool store and ambient storage

Week	Log change/day							
	'Royal Gala' skin		'Scired' skin		'Royal Gala' calyx		'Scired' calyx	
	Cool store	Ambient	Cool store	Ambient	Cool store	Ambient	Cool store	Ambient
0	0.00	0.40	0.00	0.40	0.00	0.23	0.00	0.21
1	0.46	0.15	0.31	0.32	0.16	0.12	0.14	0.10
2	0.15	0.11	0.24	0.15	-0.01	0.12	0.00	-0.10
4	-0.05	0.06	-0.02	0.00	0.06	0.03	0.04	0.03
8	0.06	0.00	0.05	-.011	-0.02	0.17	-0.00	0.00
12	0.00	0.00	0.00	0.00	0.04	-0.0	0.02	0.03
t-test	0.87		0.63		0.13		0.81	

Table 2.3: Log change/day comparison of inoculated *Listeria monocytogenes* on apples during simulated cool store and ambient shelf life (for eight days) over 20 weeks for Europe, and t-test results between the log change/day values of cool store and ambient storage.

Week	Log change/day							
	'Cripps Pink' skin		'Scired' skin		'Cripps Pink' calyx		'Scired' calyx	
	Cool store	Ambient	Cool store	Ambient	Cool store	Ambient	Cool store	Ambient
0	0.00	0.31	0.00	0.34	0.00	0.17	0.00	0.20
1	0.37	0.06	0.34	0.09	0.17	0.06	0.13	0.02
2	0.07	0.03	0.13	0.02	0.06	-0.01	0.08	-0.03
4	-0.01	0.10	-0.02	0.11	0.02	0.00	0.03	0.05
8	0.02	0.15	0.02	0.19	-0.00	0.00	0.00	0.00
12	0.02	0.00	0.03	0.09	0.01	0.00	-0.01	-0.02
16	0.02	0.00	0.00	0.00	0.02	0.13	0.03	0.16
20	0.00	0.00	0.00	0.00	0.03	0.00	0.03	0.00
t-test	0.74		0.48		0.88		0.75	

Figure 2.5 shows the β and α values for the USA and Europe's cold store simulation with the final temperature at ~0.5°C. For inactivation kinetics, the accuracy of the Weibull model was determined by the parameter of coefficient of determination, i.e. R^2. For the USA simulations, all were good fit with R^2 values of 0.95 or higher except the 'Royal Gala' calyx (which had 0.8). R^2 for all the Europe simulations were at least 0.9 showing a good fit of the Weibull model for the inactivation of *L. monocytogenes* in apples. Values for β or the shape factor remained the same for all the cultivars and the inoculation regions for both the USA and Europe simulations ($P > 0.05$). For both the cultivars and at both the regions, the shape

factor was $\beta < 1$, evidence that weak or sensitive members of the populations are destroyed, leaving behind survivors of higher resistance, resulting in a tailing phenomenon of bacterial survival curves (Buzrul et al., 2005). This meant that variable temperature, as stress in this trial, has not affected the surviving cells, hence the remainder of the cells adapted to the condition. The presence of survivors shows that the low temperature alone did not mitigate the risks from *L. monocytogenes* in cool store situations and would not be considered a mitigation step. The inactivation kinetics of *Escherichia coli* and Salmonella Typhimurium in organic carrots were also found to have a shape factor <1 at different temperatures (Liu et al., 2019). The shape factor of *L. innocua* in fresh-cut Chinese cabbage and *L. monocytogenes* in soybean sprouts was <1 as well (Alenyorege et al., 2019; Ngnitcho et al., 2018). The ability of the organism to adapt to stress at a given time probably plays some role in the tailing phenomenon as well (van Boekel, 2002).

For the USA simulations, the scale factors (α) were 1.67 and 1.46 for 'Scired' and 'Royal Gala' skin inoculations, respectively, and 1.28 and 1.31 for calyx inoculations, respectively. For Europe, α was 1.51 and 1.50 for 'Scired' and 'Cripps Pink' skin, respectively, and 1.32 and 1.34 for calyx, respectively. The change in scale factor describes the effect of storage conditions on the survival of *L. monocytogenes*. In this study, the scale factor values for inoculation on the skin was higher than the calyx inoculation, meaning skin inoculations will take less time for 1 log reduction compared with calyx inoculations (Buzrul and Alpas, 2007). There were no differences ($P > 0.05$) in either α or β calyx values between the open calyx ('Scired') or closed calyx ('Royal Gala' and 'Cripps Pink') apples, confirming that this factor did not affect the survival of *L. monocytogenes*. However, the 'Scired' skin had a significantly higher α value ($P < 0.05$) than the 'Royal Gala' in the USA trial whereas in the Europe trial, it had a similar α value to the 'Cripps Pink' skin. Comparing Figure 2.5A with Figure 2.3A show that the rapid decline in the first two weeks of storage was thus faster in

the ‘Scired’ so this cultivar might be more susceptible to the dynamic temperature conditions of the conditions in the USA simulation (Figure 2.3A). However, the more rapid decline in *L. monocytogenes* numbers on ‘Scired’ is only based on the triplicate samples at a single time point (day 7, Figure 2.3A) and a more detailed study of the inactivation over the first two weeks would be needed to draw firm conclusions. Also, work is required on the differences of surface morphology of the 3 cultivars used in this study, which could provide information of the susceptibility of the bacteria towards these cultivars.

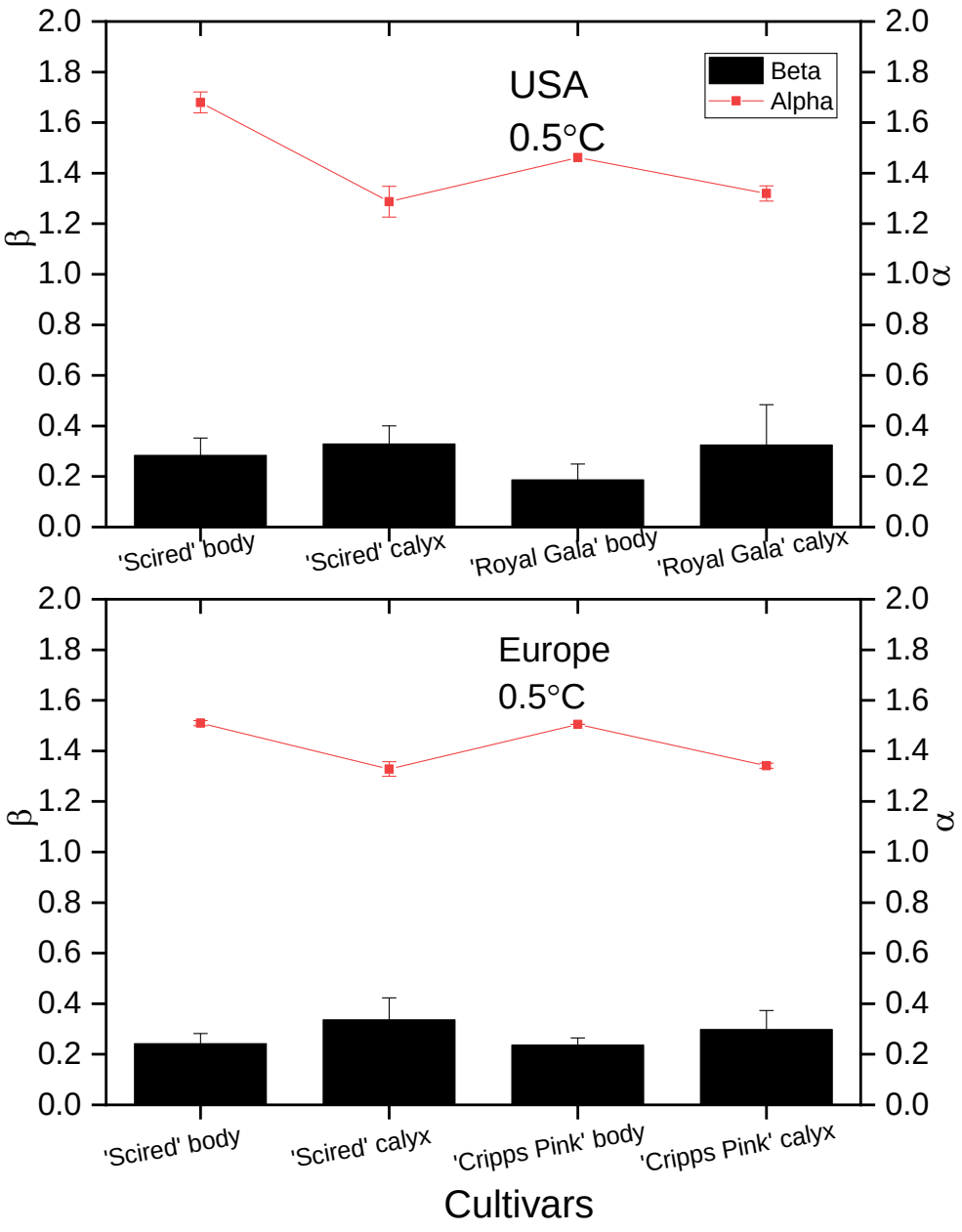

Figure 2.5: Inactivation kinetics of inoculated *Listeria monocytogenes* on the skin and in the calyx, using different apple cultivars, targeting USA and Europe simulated transport in the lab for 12 and 20 weeks, respectively. Weibull parameters are shown having β and α as the shape and scale parameter, respectively. Error bars are represented as standard deviations.

In Figures 2.6A and 2.6B, addition to survival characteristics of *L. monocytogenes* over selected storage conditions, the selected quality attributes were also assessed. The change in

firmness and SSC of apples cold stored for 12 weeks for the USA and 20 weeks for Europe is shown in Figures 6A and 6B, respectively. The average firmness of 'Scired' and 'Royal Gala' remained almost constant, finishing at 7.8 and 6.8 kgf, respectively, for USA simulations. For Europe as well, the apple firmness stayed consistent for both the cultivars, with average firmness for 'Scired' and 'Cripps Pink' at 7.8 and 7.7 kgf, respectively. For both USA and Europe, the average firmness of apples did not change during storage because both apple cultivars were industrially treated with 1-Methylcyclopropene (1-MCP). 1-MCP acts as an ethylene antagonist by inhibiting ethylene and therefore improving firmness retention in apples (Rupasinghe et al., 2000). With no variation in firmness with time, correlation data could not be used to evaluate whether fruit firmness played a role in the bacteria's survival ($R^2 = 0.0352$). However, there is a possibility that the softer skin of the 'Royal Gala' apples may have contributed to the occasions when more rapid inactivation of *L. monocytogenes* was observed on the skin of this variety compared with that on 'Scired' (Figure 2.3A).

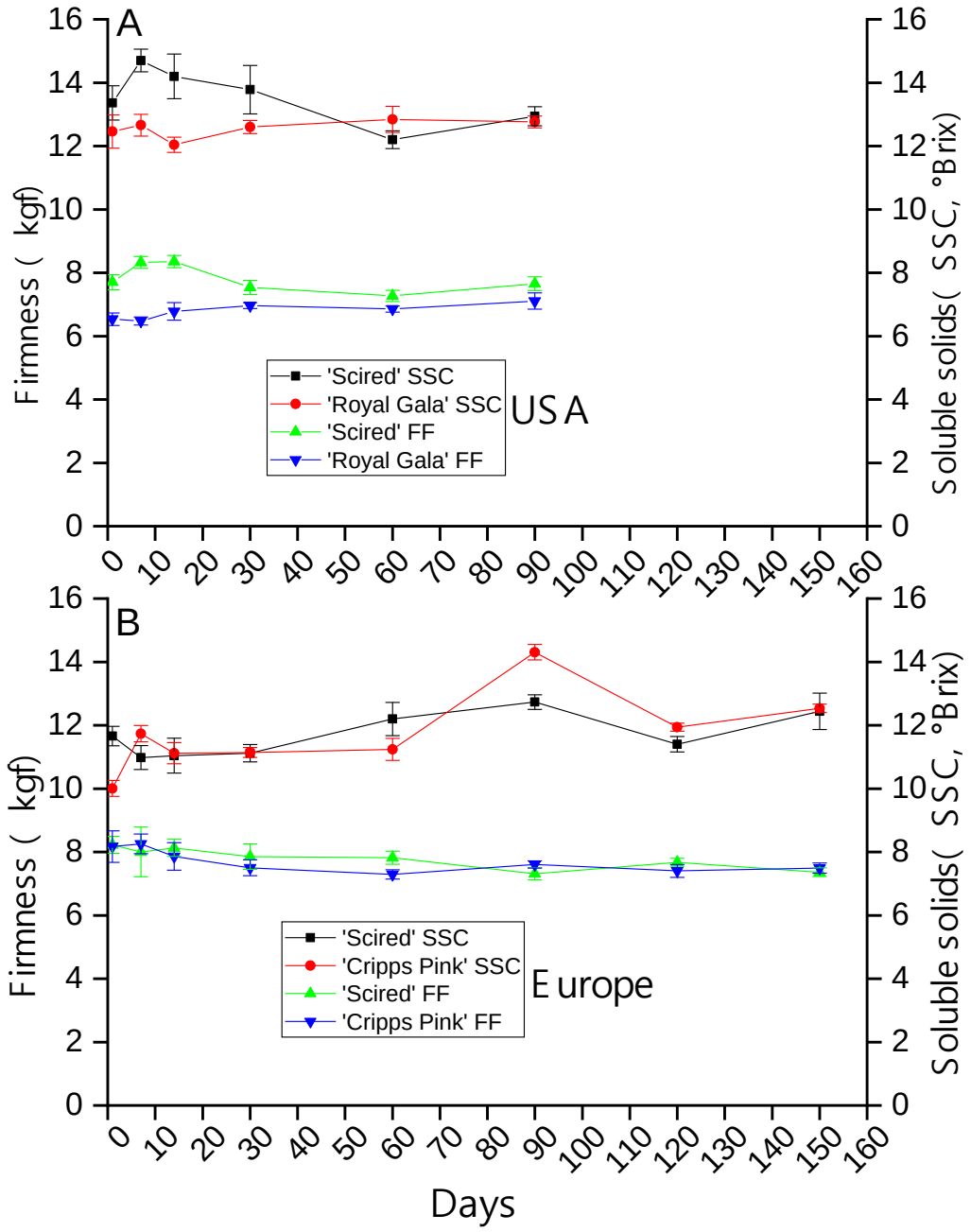

Figure 2.6: Firmness (kgf) and soluble solids concentration (SSC) of apples during laboratory simulated storage for commercial shipments for 12 weeks for the USA (A) and 20 weeks for Europe (B), respectively. Error bars are represented as standard deviations.

Throughout the storage period, SSC had an average of 13.5 and 12.6 °Brix for 'Scired' and 'Royal Gala', respectively, for the USA, and 11.7 °Brix for both 'Scired' and 'Cripps Pink'

for Europe. As found for fruit firmness, no correlation of SSC and *L. monocytogenes* counts was able to be established ($R^2 = 0.0453$) but the higher °Brix of the 'Scired' in the US may be related to the more rapid decline in *L. monocytogenes* numbers in the first two weeks of this study. It should be important to note that the comments on the relationship between °Brix and *L. monocytogenes* requires more data to verify the claims.

SPI stayed the same (SPI=6) throughout the USA study, which means that all the starch had previously been converted into sugar. For the Europe study, the SPI started at 4, which means there was still some starch present, but fruit ripened and SPI reached 6 at 4 weeks (data not shown). There is nothing to suggest that this ripening during cold storage affected the survival of *L. monocytogenes*.

2.4 Conclusion

This study demonstrated the effect of long-term storage on *L. monocytogenes,* showing consistent effects across all the cultivars and two seasons for simulated export conditions for two key New Zealand markets. Although *L. monocytogenes* is capable of growing at refrigeration temperatures, the study provided evidence that surfaces of whole apples present an unfavourable environment for growth under typical cold storage conditions and numbers of *L. monocytogenes* invariably declined during storage. In both simulated storage studies, more than 5 log reductions occurred on apple skin surfaces during storage. The ambient shelf-life studies showed that, if anything, retail storage is likely to result in further reductions in *L. monocytogenes* numbers. This information will provide added food safety assurance for apple producers who typically apply other measures (washing etc.) to attempt to eliminate any environmental *L. monocytogenes* that may be present on apple surfaces. If any remain present in low numbers, they are highly unlikely to survive normal export shipping regimes. However, because this is the first-ever dynamic storage study done on *L. monocytogenes*, more data is required to make comment on this precisely.

However, in all cultivars used in this study, regardless of whether open or closed calyx, once *L. monocytogenes* enters the calyx (or perhaps other hidden parts such as the stem-end), its survival was enhanced compared with survival on the main skin of apples. During the export shipping simulations, only 2–3 log reductions were observed in apple calyces. The risk of causing illness is, therefore higher if contaminated apple cores are eaten or if *L. monocytogenes* harbouring in the core is allowed to cross-contaminate a food ingredient that is more conducive to growth, as is suspected of having occurred in the 2015 US caramelised apple outbreak. Further, although good reductions in numbers were observed in this study, the possibility that the organisms have not been inactivated but have gone into a VBNC state cannot be ruled out and should be further investigated.

It should also be noted that the current study was done under simulated controlled conditions, where the temperature declined from 6 to 0.5°C (for the USA) and 2 to 0.5°C (for Europe), which is close to commercial reality. More work is required to know the effect of static temperatures on the survival of *L. monocytogenes*, particularly during the first two weeks of storage.

2.5 Acknowledgements

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Preface to Chapter 3

Chapter 2 quantified the nature of dynamic temperatures in the international sea-freight supply chain and the fate of *L. monocytogenes*. Due to the number of steps involved post-packhouse for the domestic supply chain, the temperatures are more variable than the international supply chain. The literature revealed that trucking, distribution centres, and grocery store operations are critical phases of the supply chain that have somewhat been overlooked in food safety research. There is a scarcity of information on the domestic supply chain and the fate of *L. monocytogenes*. This chapter investigated monitoring of the variable supply chain temperatures and simulating the retrieved temperatures in the laboratory to examine fate of *L. monocytogenes.*

Chapter 3: The effect of dynamic temperatures on *Listeria monocytogenes* in the domestic apple supply chain

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Abstract

A vital aspect of an optimal fruit and vegetable supply chain is the maintenance of low temperatures to maintain fruit quality, as well as minimising the proliferation of foodborne pathogens. If any produce is contaminated, variable temperatures in the supply chain could allow an opportunistic, psychrotrophic foodborne organism such as *Listeria monocytogenes* to grow and persist longer. To understand the fate of *L. monocytogenes* on fresh produce like apples in the domestic supply chain, a series of studies were conducted to simulate the dynamic nature of the supply chain. Temperatures were recorded on two occasions for every step of three New Zealand domestic supply chains, i.e., transportation, distribution centre (DC), and retail. The observed temperatures along the supply chain were simulated in a series of laboratory trials, using open ('Scired' for all the supply chains) and closed ('Cripps Pink' for supply chain A, and 'Royal Gala' for supply chains B and C) calyx cultivars of apples. The cultivars were separately inoculated in the calyx and on the apple body with a cocktail of 10^8 cells of seven strains of *L. monocytogenes. L. monocytogenes* was enumerated in all the apple samples on days 0, 1, 4, 7 and 10 for supply chain A and days 0, 1, 2, 3, 4, 5 for supply

chains B and C, respectively. At the end of the study, for supply chain A, the final log changes in the calyx for 'Scired' and 'Cripps Pink' were reductions of 1.65 and 1.76 $\log_{10}$ MPN/apple, respectively, whereas on the body the reductions were 2.62 and 2.73 $\log_{10}$ MPN/apple, respectively. For supply chains B and C, the total log reductions in the calyx for 'Scired' and 'Royal Gala' were 2.69 and 2.43 $\log_{10}$ MPN/ apple for supply chain B, and 2.13 and 2.03 $\log_{10}$ MPN/apple for supply chain C, respectively. In contrast, the total log reductions on the body were 3.00 and 3.25 $\log_{10}$ MPN/apple for supply chain B and 3.25 and 4.69 $\log_{10}$ MPN/apple, respectively for supply chain C. At all the different dynamic temperatures, no growth was observed in any supply chain studies, either in the calyx or on the body. For all three supply chains, the concentrations of surviving *L. monocytogenes* were significantly higher in the calyx than on the body for all the cultivars ($P < 0.05$). Inactivation kinetics using the Weibull model showed that for all the supply chain simulations, the shape factor or β was < 1, which means that low-resistance bacteria died during the study but others survived, called the tailing effect. The results give crucial information and assurance to the postharvest handlers on their operating temperatures.

Keywords: Dynamic temperatures, apple skin and calyx, inactivation kinetics, distribution centres, trucking, grocery stores.

3.1 Introduction

The New Zealand fresh produce supply chain is a continuum of processes from harvesting through grading, packaging, storage, transporting, distribution, and retail stores. In the process, proactive temperature management of horticultural produce is required (Aitken et al., 2006). Maintaining the temperature is crucial to the fresh produce industry, as it provides consumers with good quality product (Dallaire et al., 2006). Failing to do so could increase

spoilage and postharvest decay and promote the growth of foodborne pathogens, resulting in foodborne illnesses and product recalls (Colás-Medà et al., 2017; Mercier et al., 2017).

Listeria monocytogenes has become one of the prominent food safety concerns in the fresh produce industry, including the apple supply chain worldwide. Eating food contaminated with *L. monocytogenes* may lead to listeriosis, particularly in immunocompromised people (Nangul et al., 2021; Zhu et al., 2017). *L. monocytogenes* can also cause illness in a healthy population when the consumed food's bacterial concentration is as high as 1.9×10^5 CFU/g to 1.2×10^9 CFU/g, depending on the serovar type (FSANZ, 2013). Listeriosis is relatively rare but has a high fatality rate of up to 30% (Scallan et al., 2011). *L. monocytogenes* can grow in a variety of fresh produce (Huang et al., 2019), at temperatures from frozen (-1.5°C), ambient (20°C) to hot (45°C), which means that this bacteria could survive and grow throughout the supply chain (Pinton et al., 2020). *L. monocytogenes* survival on any fresh produce is influenced by environmental parameters like temperature in the packhouse and during distribution (Kuttappan et al., 2021).

Temperature directly affects the rate of respiration, transpiration, ethylene, internal quality and proliferation of microorganisms (Goedhals-Gerber and Khumalo, 2020). Apples generally have an optimal storage temperature of 0 - 2°C (Mercier et al., 2017) and the postharvest apple cool chain should be unbroken through storage, sorting, grading, packaging, handling and transport to market (Nissen et al., 2018). Because the apple supply chain consists of many service providers; communication, timely implementation of process and practices, and a good understanding of practices to handle and store apples is crucial for an effective supply chain (Nissen et al., 2018). New Zealand's domestic apple supply is a multistep process, where the fruit is transferred from the packhouse to display cabinet in a grocery store.

Figure 3.1 shows the typical New Zealand domestic supply chain, post-packhouse, where low-temperature management is critical.

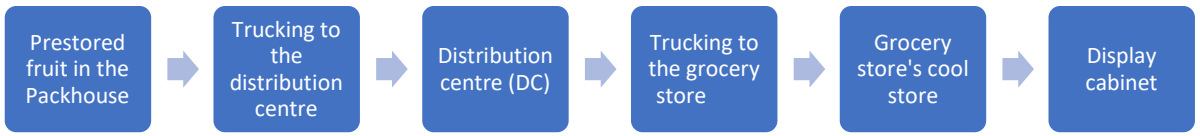


Figure 3.1: A simplified New Zealand domestic postharvest supply chain for fruits and vegetables.

Although the importance of temperature management is widely accepted, implementation in the apple supply chain is not as straightforward as one might expect. Post-packhouse, temperature fluctuations (± 5°C) have been reported during transportation, retail storage, and display cabinets of grocery stores (Ndraha et al., 2018). Some legislative rules require food businesses to have specific requirements for vehicles, transportation equipment and transportation operations to preserve the safety and quality of food (e.g. use of refrigerated transportation trucks for climacteric fruits - USFDA, 2017). Despite these legal requirements, effective temperature management remains a challenge in practice (Ndraha et al., 2018), and the lack of data guides the need to understand the microorganisms better.

As the New Zealand domestic supply is a multistep process (Fig. 3.1), there are many opportunities for breaks in the cool-chain, e.g. during trucking, distribution centres (DC) and grocery stores. Trucking is the standard mode of delivering fresh produce from one place to another. Generally, refrigerated trucks aim to keep the temperatures below 1°C to slow down the proliferation of foodborne pathogens and keep the freshness of the produce. However, temperature abuse during trucking is common; e.g. the temperature of strawberries during a four-day shipment from California increased from 1.5°C to 6°C (Pelletier et al., 2011). The

temperature of fresh-cut lettuce measured during different seasons increased to above 10°C when the truck was loaded or unloaded (McKellar et al., 2014). Breaking the cold chain sometimes does occur in the postharvest supply chain (Dallaire et al., 2006), potentially resulting in growth of opportunistic and psychrotrophic bacteria.

The distribution centre (DC) is a critical part of many cold chain management systems after transportation by truck. It allows for sorting and combining shipments received from many suppliers and scheduling their dispatch (Mercier et al., 2017). Even if transportation trucks maintain the desired transit temperature, DCs may or may not have storage rooms for all the different temperatures needed for all the commodities. Some of the commodities can be held at a temperature that can be detrimental to the life of the fresh produce (e.g. in a trial apples and tomatoes were kept at 5°C for short period, Tokala and Mohammed, 2021a). There is not enough literature on DCs and the fate of the fresh produce at which DC temperatures operate. The only recent study that has been reported investigated 18 DCs in the USA and found *L. monocytogenes* on floor surfaces and shipping docks. The study found that any change in the situations or conditions could impact the food safety risks to fresh produce (Townsend et al., 2022).

Another aspect of a DC is that fruits and vegetables are held in storage rooms before processing or re-packing. The DC storage rooms' temperature and humidity are controlled to reduce respiration and dehydration, although sometimes at higher than recommended temperatures for any specific cultivar. As a result of high temperatures for any specific cultivar, the psychrotrophic nature of bacteria like *L. monocytogenes* enables the pathogen to become established on condenser coils and units, thereby representing a potential continuous source of contamination with distribution throughout the storage room facilitated by airflow (Ndraha et al., 2018; Warriner and Hasani, 2020). The airflow also results in organic debris

becoming trapped on meshes and fans that can sustain the formation of biofilms that can be long-lived and complex to remove (Warriner and Hasani, 2020).

After DC, retail is the last link of the cold chain infrastructure. The commodity transported to different locations often does not benefit from the cold chain until it is maintained at the recommended temperatures at retail. Even then, cooling at the retail level is often neglected, and, in most cases, fresh fruits and vegetables are displayed at ambient temperatures, exposing them to temperatures as high as 30°C–35°C in tropical countries (Tokala and Mohammed, 2021b). When fresh produce reaches the grocery store, it is placed either in a display cabinet or in a refrigerated storage room. Time-temperature measurements indicate that display cabinet temperatures often rise above the desired limit (Mercier et al., 2017), e.g., a survey on the temperature of fruits and vegetables for 28 retail stores in Canada found the average temperature at 8°C, significantly above the recommended temperature of 4°C (LeBlanc et al., 1996). Literature on retail market and display cabinet on *L. monocytogenes* in the United States between 1988 and 2020 detected *L. monocytogenes* in fresh produce. This data showed that *Listeria* has been persistent before and after the zero-tolerance policy in the USA. This makes *Listeria* a consistent issue in the retail fresh produce (Townsend et al., 2021). If any produce is contaminated with any foodborne pathogen, the higher temperatures at retail could allow psychrotrophic foodborne organisms like *L. monocytogenes* to grow and survive longer. New Zealand has recorded no data for the persistence of *L. monocytogenes* in the retail or grocery stores, hence it is important to do a challenge study that sheds light on the domestic apple supply and *L. monocytogenes* persistence.

The current research investigates the risks of *L. monocytogenes* persistence and/or growth during post packhouse distribution period under the temperature conditions found in the New Zealand domestic apple supply chain. No data was previously available to show whether

dynamic environmental conditions (temperature as the current focus) in the domestic supply chain result in pathogen proliferation and could contaminate the fresh produce postharvest, i.e. postharvest handling, distribution, and retail (Dallaire et al., 2006; Gorny, 2005).

Supply chain temperatures are dynamic. Research on simulating the international supply chain for apples showed that temperature plays a crucial role in the *L. monocytogenes* survival (Nangul et al., 2021). However, the focus of the paper was the international transport supply chain and the research was done over 3 and 6 months. For the domestic supply chain, due to the many steps to reach the retail stores (Fig. 3.1), the temperature profile is more variable than the international supply chain. It is unclear if *L. monocytogenes* survives or even grows under the dynamic temperature conditions that reflect reality during domestic supply chain transport, due to increased number of steps in the domestic supply chain (Nangul et al., 2021). There is little quantitative data available on the level and fate of microorganisms through the entire postharvest supply chain, thus limiting the ability to adapt recommended management practices to any commodity (Dallaire et al., 2006; Doering et al., 2009; Zoellner et al., 2018).

It is important to note that the postharvest packing of apples does not have any kill step (such as pasteurisation) that can inactivate *L. monocytogenes* (Pietrysiak et al., 2019). Hence, studying the inactivation of the bacteria under the dynamic condition of a domestic supply chain is crucial, since, under stress, microorganisms decline in a non-linear fashion (Kahraman et al., 2017).

Therefore, the objective of this study was to determine the effect of variable temperatures of the domestic supply chain on the survival or growth of *L. monocytogenes* by:

A. Recording temperatures of the domestic supply chain, post-packhouse during two seasons;

B. Determining *L. monocytogenes* survival in open and closed-calyx cultivars on two regions of apple (body and calyx) during the supply chain;
C. Determining the inactivation kinetics of *L. monocytogenes* for each cultivar on each part of the apple.

3.2 Materials and methods

3.2.1 Domestic supply chain conditions

The temperature profile of a typical domestic apple supply chain was recorded during two seasons (2019, 2020) in order to simulate the fate of *L. monocytogenes* at those temperatures. The supply chain, with end-point grocery stores, was selected based on the variety of apples a grocery store received. Because most apple-growing regions are located in the North Island of New Zealand, a supply chain concentrated in the North Island was selected. Real-time temperature and humidity loggers (Cydiance, China) were used for this temperature study. The temperature profiles of the apples were monitored post-packhouse, which included trucking to the distribution centre (DC), time in the DC, trucking to the grocery store, retail coolstore, and finally to the display cabinet of the grocery store. After finishing the exercise, the temperature profiles were retrieved from the data loggers. Then, using two apple cultivars, those retrieved temperatures were simulated in the lab to study the survival behaviour of *L. monocytogenes*.

3.2.2 Fruit

Commercially graded unwaxed apples (*Malus domestica*), 100 count size for 18 kg carton, without any bruising, cuts or scars, were obtained from a commercial New Zealand packhouse. The fruit had been through conventional packhouse treatments such as high pressure washing and the use of sanitisers in flumes. In 2019, ‘Cripps Pink’ and ‘Scired’ were selected as they represent closed and open calyx cultivars respectively, whereas ‘Royal Gala’ and ‘Scired’ were used as closed and open calyx cultivars, respectively, in 2020.

3.2.3 L. monocytogenes strain selection

Six genetically variable *L. monocytogenes* strains from horticultural sources were obtained from The New Zealand Institute for Plant and Food Research Limited's (PFR) culture collection (PFR46G06, PFR41I04, PFR40I07, PFR46E10, PFR41F08, PFR41H05). In addition, one clinical strain (Scott A) was added to make a seven-strain bacterial cocktail. The details of the lineages and its sequence types have been described previously (Nangul et al., 2021, Chapter 2).

The seven isolates were grown in tryptic soy-yeast extract broth at 37°C. Genomic DNA was extracted using a bacterial genomic DNA extraction kit (Geneaid Presto™ Mini gDNA bacterial kit, Geneaid Biotech Ltd., New Taipei City, Taiwan) according to the manufacturer's protocol. The DNA was sent for whole genome sequencing by Westmead Institute for Medical Research and Centre for Infectious Disease and Microbiology, Sydney, Australia (Nangul et al., 2021).

3.2.4 Preparation of Inoculum

The inoculum was prepared according to the protocol described previously (Nangul et al., 2021). *L. monocytogenes* individual strain purity was confirmed by streaking onto tryptic soy agar with 0.6% added yeast extract (TSAYE) (BD, Becton, Dickinson & Company, USA) and then onto selective media *Listeria* CHROMagar™ plates (CHROMagar™, Paris, France) and checking for blue colony appearance and uniformity. Pure cultures were then grown in tryptic soy broth with 0.6% added yeast extract (TSBYE) (BD, Becton, Dickinson & Company, USA) for 48 h at 37°C to achieve a stationary phase. Those strains were pelletised by centrifuging at 4000 rpm (3220 *g*) (Gyrozen model 1736R, Seoul, South Korea) for 10 min at 4°C. The resulting pellets were washed twice with 0.1% peptone (Bacto™, BD Biosciences, USA) and re-centrifuged. Equal amounts of each *L. monocytogenes* strain were

combined to make a seven-strain cocktail with a final concentration of 10^8 CFU/mL in 0.1% peptone that was used for apple inoculation.

3.2.5 Inoculation of apples

Before inoculation for all the challenge studies, unwaxed apples were moved from cold storage (0.5°C) and held at room temperature (~20°C) overnight to equilibrate the apple temperature. There are many ways in the apple supply chain that an apple could become contaminated. It could be by a splash of contaminated water, coming in contact with contaminated surfaces such as packhouse contact surfaces or being immersed in contaminated water such as flumes used for moving apples in a packhouse (Nangul et al., 2021). Spot-inoculation method on apples was used to simulate the splash of contaminated water. For each cultivar, apples were inoculated at two regions: (i) inoculation in the calyx, where one 50 µL aliquot of *L. monocytogenes* cocktail was pipetted into the calyx of an apple, and (ii) inoculation on the body of an apple, where two drops, each of 25 µL, were pipetted on the equatorial region. All inoculated apples were air-dried for 3 h in a biosafety class II cabinet at room temperature until visibly dry.

3.2.6 Post-packhouse supply chain simulation and sampling

After inoculating the apples, the fruit were placed in polyethylene lined cardboard boxes (to maintain humidity and prevent water loss) and then stored in a temperature-controlled room to simulate the temperatures from the domestic supply chain temperature profiles (Figure 3.2).

A freezer/cold store with a capacity to control temperatures between -5°C to +30°C was used to simulate the temperature profile. The 8 KW refrigeration system was controlled by a temperature controller (Yudian Automation Technology Co., Ltd., Hong Kong) and LabVIEW software (v2019, National Instruments Inc., USA) to simulate the temperature

profile. The dead-band range was set to 0.8°C, and the auxiliary output high deviation level was set to 0.9°C. The auxiliary output controls the compressor (Nangul et al., 2021).

The temperature and humidity were monitored at 1 h intervals using a real-time temperature and humidity logger (Cydiance, Shanghai, China). Maxim type-L-iButton temperature loggers (Maxim Integrated, USA) were used as a backup at 30-min intervals. The fruit were sampled after 0, 1, 4, 7 and 10 days for supply chain A in 2019 and 0, 1, 2, 3, 4, 5 days for supply chains B and C in 2020. The different sampling regimes were used to simulate the transport durations of different supply chains. At each time-point, three sets of three apples of each point of inoculation were sampled from each cultivar per inoculation.

3.2.7 Microbial enumeration of apples

Using the most probable number (MPN) method which allowed the detection of stressed organisms (Osborne and Bremer, 2002), three sets of three apples of each inoculation method on the body or calyx were quantitatively assessed for *L. monocytogenes.*

The protocol for MPN methodology for both body and calyx inoculation was sourced from (Nangul et al., 2021). For samples inoculated on the body, three apples were placed into a sterile bag with 400 mL of Buffered *Listeria* Enrichment Broth (BLEB) (Acumedia, Lansing, Michigan, USA) and hand massaged for 2 min. Triplicate 2 mL aliquots of BLEB wash solution from each bag were transferred into 15 mL centrifuge tubes (Cellstar™ sterile, Germany). Triplicate 200 µL BLEB aliquots were dispensed in microtiter well plates (Greiner, Germany), followed by 10-fold serial dilutions. Aliquots and the original bag were then incubated for 48 h at 30°C for enrichment. After 48 h, 2 µL aliquots of the enriched BLEB from each bag, tube, and microtiter wells were plated onto pre-gridded selective CHROMagar™ *Listeria* plates incubated for 48 h at 37°C for detection of *L. monocytogenes*. Blue colonies were recorded as positive readings for *L. monocytogenes*.

For samples where the inoculation was at the calyx, the core of the apple was removed with a sterile cork-borer (16-mm diameter), weighed, and homogenised in a laboratory stomacher (Smasher, AES Chemunex, AES Laboratory, France) for 2 min in BLEB (1:10). This was followed by the same enumeration protocol used for the skin inoculations described above. MPN values were calculated using the Bacteriological Analytical Manual (BAM) spreadsheet (Blodgett, 2010). All results were expressed as $\log_{10}$ MPN per apple with a detection limit of 1.37 $\log_{10}$ MPN per apple from the tubes and wells and presence/absence in three apples from the bags.

3.2.8 Inactivation kinetics

L. monocytogenes inactivation kinetics during the domestic supply chain simulation of apples were evaluated based on the Weibull model. The inactivation kinetics based on the Weibull model assumes that all the microorganisms rarely die simultaneously (linear) when exposed to an agent but follow non-linear kinetics (van Boekel, 2002). Experimental data were fitted in decimal logarithmic form as follows:

$$logNt/N_0 = - b \times t^{\beta} \quad (1)$$

where N (t) is the number of microorganisms surviving after the storage simulation time *t* and N_0 is the initial number of microorganisms. The *b* parameter ($min^{-\beta}$) is defined as:

$$b = 1/2.303 \times (1/\alpha)^{\beta} \quad (2)$$

Where α (day^{-1}) is the coefficient in the Weibull distribution, known as the scale parameter, and β is the shape parameter. If $\beta < 1$, it shows that the remaining cells can adapt to the stress, i.e. the remaining cells are resistant to change (also known as a tailing effect). If $\beta > 1$, the remaining cells become damaged because of the treatment and possibly cannot revive themselves (also known as a shoulder effect). The model describes first-order kinetics in rare cases when $\beta = 1$ (Bozkurt et al., 2016; Buzrul et al., 2005; van Boekel, 2002).

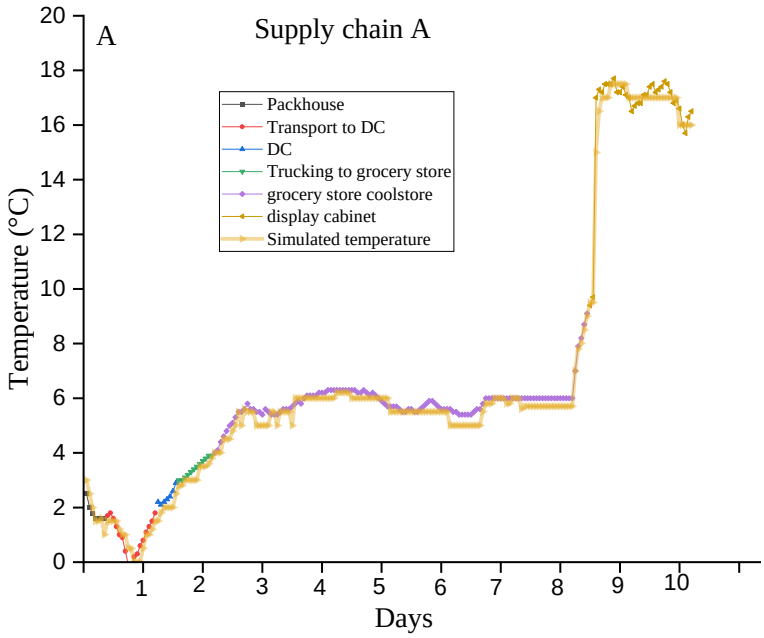
3.2.9 Statistical analysis

Microbial data (log_{10} MPN/apple) and inactivation kinetics data were analysed using GenStat (version 18th; VSN International Ltd), using a two-way analysis of variance (ANOVA) followed by post-hoc Tukey's test. Results are presented as means with standard deviation in the figures, with $P < 0.05$ considered statistically significant. All the figures were prepared using the Origin software 2021b (OriginLab Corporation, Northampton, USA). For inactivation kinetics, non-linear regression analyses were performed using the SPSS statistical package version 26 (IBM Corporation, Armonk, NY, USA).

3.3 Results and discussions:

3.3.1 Transport simulation of apples

Figures 3.2A, 3.2B, and 3.2C show the temperatures during the three scenarios recorded during the transport journey of the domestic supply chains A, B and C of apple, respectively. In Fig. 3.2(A), supply chain A was for 10 days, whereas in Fig. 3.2(B) and 3.2(C), supply chains B, and C were for five days.



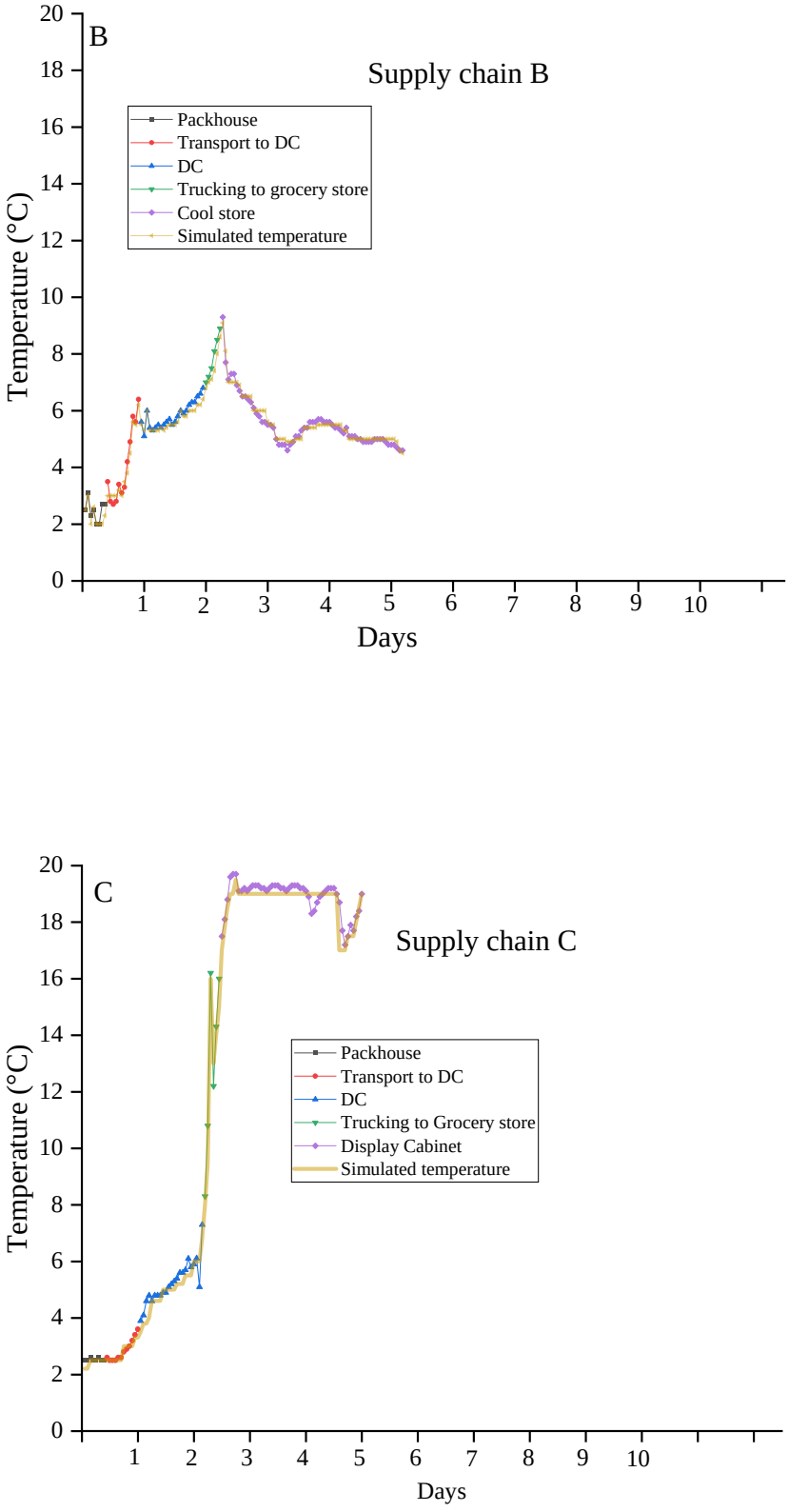


Figure 3.2: Recorded temperature profiles of apples in the domestic supply chains A, B, and C in New Zealand and the temperature profiles (yellow lines) simulated in the laboratory.

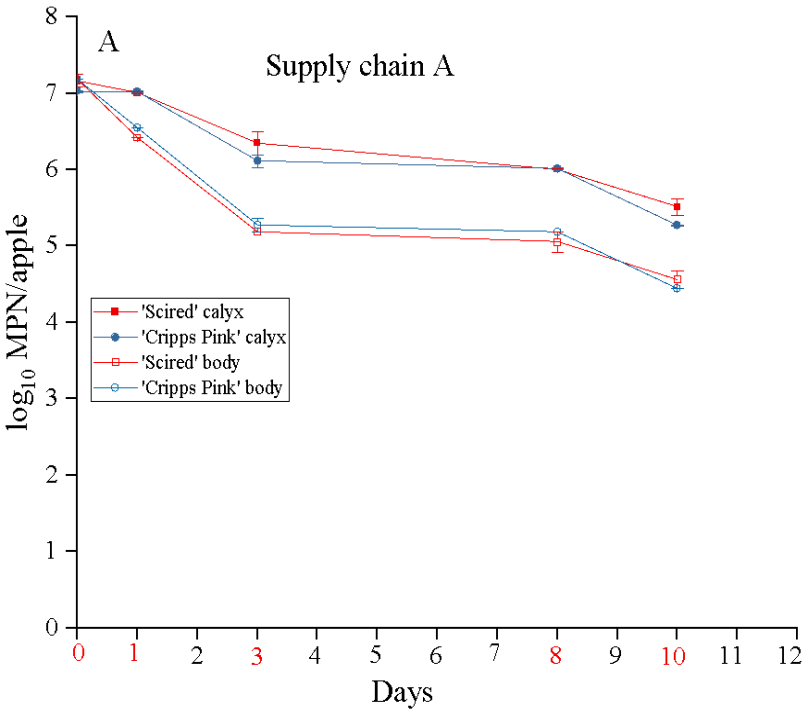
The critical temperature fluctuations (mean) (°C) observed in all the supply chains are presented in Table 3.1 below.

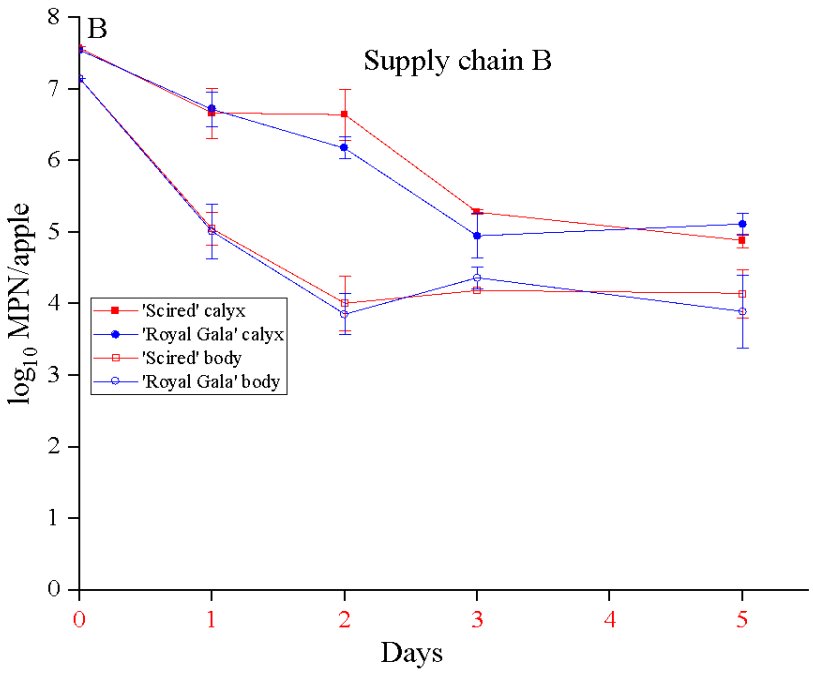
Table 3.1: Mean temperatures of various steps in the three domestic apple supply chains, where each supply chain shows a different scenario.

Locations	*Supply chain A*	*Supply chain B*	*Supply chain C*
	Mean temperatures (°C)		
Packhouse	2.3	2.5	2.5
Trucking to DC	2	6	3.8
DC	2.5	6	6
Trucking to the grocery store	4	9	16
Grocery store coolstore	6	5	NA
Display cabinet	17	NA	19

3.3.2 Effect of dynamic temperatures of the simulated supply chain on L. monocytogenes survival

Temperatures for each supply chain were simulated in the lab, and the concentrations of *L. monocytogenes* found on the apples during the storage is shown in Figure 3.3 A, B, C.


A
Supply chain A
log10 MPN/apple
Days
'Scired' calyx
'Cripps Pink' calyx
'Scired' body
'Cripps Pink' body


B
Supply chain B
log10 MPN/apple
Days
'Scired' calyx
'Royal Gala' calyx
'Scired' body
'Royal Gala' body

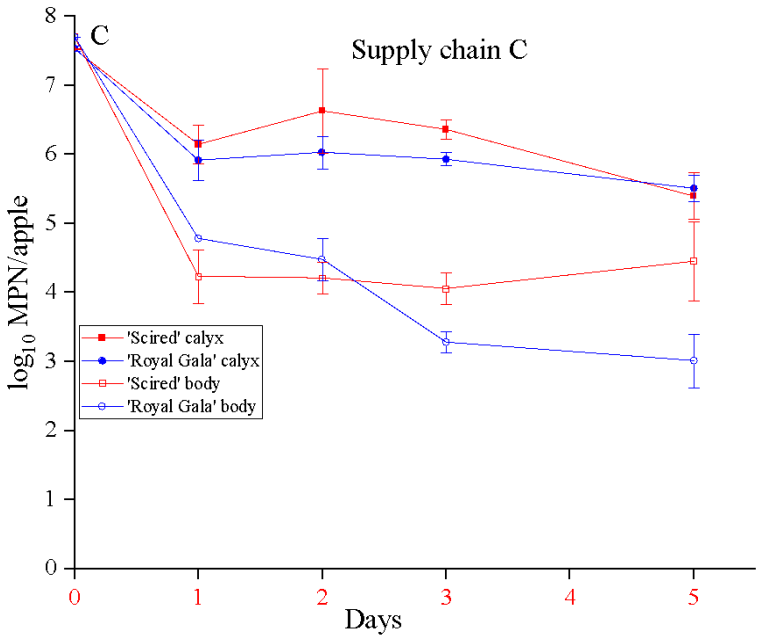


Figure 3.3: Survival of inoculated *L. monocytogenes* on the body and calyx of apples during laboratory-simulated storage for three commercial, domestic supply chains on open and closed calyx cultivars. The days with red colour on the x-axis show the sampling time-points.

The initial titres of *L. monocytogenes* inoculum applied to the apples were 10^8 CFU/mL for all the supply chains. After four hours of inoculation, the mean recovered titres for Supply chain A were 7.16 and 7.03 $\log_{10}$ MPN/apple for 'Scired' and 'Cripps Pink' calyces, respectively. For 'Scired' and 'Cripps Pink' body inoculations, the mean recovered titres were 7.18 and 7.08 $\log_{10}$ MPN/apple. For supply chain B, the mean recovered titres were 7.57 and 7.55 $\log_{10}$ MPN/apple for 'Scired' and 'Royal Gala' calyces, respectively, and 7.14 and 7.14 $\log_{10}$ MPN/apple for 'Scired' and 'Royal Gala' body inoculations. For supply chain C, the mean recovered titres for 'Scired' and 'Royal Gala' calyces were 7.53 and 7.53 $\log_{10}$ MPN/apple, respectively, and 7.70 and 7.70 $\log_{10}$ MPN/apple for 'Scired' and 'Royal Gala' body inoculations. The slight drop in *L. monocytogenes* titres after inoculation could be due to organisms dying during the drying process, or the inability of the hand massage method to remove all organisms from the apple surfaces, or differences in the accuracy of the two

methods (plate counts compared with MPN). The former is likely because hand massage would be expected to recover better from skin inoculations than calyx (Nangul et al., 2021). The other reason behind the drop in *L. monocytogenes* titre on the body could be the drying process causing cell damage, but drying is likely to be slower in the calyx, resulting in less damage.

At the end of the study, the final *L. monocytogenes'* concentrations for each supply chain are shown below in Table 3.2.

Table 3.2: Final concentrations and log reductions of *L. monocytogenes* for each of cultivar in the various inoculation regions for three apple supply chains.

Supply chain	**Cultivar**	**Inoculation Region**	**Initial titre**	**Final titre**	**Log reduction**
			(log_{10}MPN/apple)		
A	'Scired'	Calyx	7.16	5.51	1.65
	'Cripps Pink'		7.03	5.27	1.76
	'Scired'	Body	7.18	4.56	2.62
	'Cripps Pink'		7.08	4.45	2.63
B	'Scired'	Calyx	7.57	4.88	2.69
	'Royal Gala'		7.55	5.11	2.44
	'Scired'	Body	7.14	2.69	4.45
	'Royal Gala'		7.14	2.43	4.71
C	'Scired'	Calyx	7.53	5.4	2.13
	'Royal Gala'		7.53	5.51	2.02
	'Scired'	Body	7.7	4.45	3.25
	'Royal Gala'		7.7	3.01	4.69

Overall, no growth of *L. monocytogenes* was observed for any of the three supply chains studied with their different scenarios. This study agreed with the previous work (Nangul et al., 2021; Ryser et al., 2019; Sheng et al., 2017) where no growth was observed at different and dynamic temperatures and different storage periods. Under non-dynamic commercial

temperatures, some studies also investigated other commodities (Kroft et al., 2022). For fresh produce such as blueberry, grapes, mangoes, *L. monocytogenes* showed no growth at abused temperature conditions of more than 20°C (Kroft et al., 2022). However, the decline in *L. monocytogenes* concentration was insufficient to significantly reduce the food safety risk, especially for immunocompromised consumers (Buchanan et al., 2017; Kroft et al., 2022). The observed decline in *L. monocytogenes* was contrary to some previous research (Danyluk, 2017; Kuttappan et al., 2021), which could be due to changes in the experimental parameters, commodity cultivar, or strains used (Kroft et al., 2022). Also, every fresh produce commodity has different native microbial communities, which could shift or change during postharvest processing and storage and may promote or hinder the growth of *L. monocytogenes* (Kroft et al., 2022; Liu et al., 2016; Salazar et al., 2016a).

For all three supply chains and cultivars, there appears to be a small number of persister cells that survive the supply chain. However, the concentrations for surviving *L. monocytogenes* were higher in the calyx than on the body ($P < 0.05$). This result agreed with the previous work (Macarisin et al., 2019; Nangul et al., 2021), suggesting that bacteria survived better in the calyx. That *L. monocytogenes* did not survive on the body might be due to the unavailability of nutrients required for survival. The bacteria may be well protected and hidden in the crevices in the calyx, which could act as a harbour site for bacterial attachment (Nangul et al., 2021; Pietrysiak and Ganjyal, 2018).

The possible presence of viable-but-non-culturable cells (VBNC) could also explain higher log reductions on the body of the apple compared with the calyx. Conventional enrichment techniques cannot detect VBNC cells as they have lost the ability to develop colonies on selective laboratory media. VBNC cells enter that state to survive and conserve energy (Li et al., 2014) due to starvation, growth outside the normal temperature, and increased or decreased osmotic pressures (Oliver, 2010). Regardless of their ability to grow in the growth

medium, VBNC cells differ from dead cells because dead cells have a damaged membrane that cannot retain chromosomic and plasmid DNA, while VBNC cells have an intact membrane containing undamaged genetic information (Li et al., 2014). VBNC cells are metabolically active, whereas dead cells are metabolically inactive (Besnard et al., 2000; Lleò et al., 2000). Even after one year of entering the VBNC state, the ATP level in *L. monocytogenes* was high (Lindbäck et al., 2010). VBNC can transcribe and produce mRNA, whereas this ability is not present in dead cells (Lleò et al., 2000). A continuous uptake of amino acids into proteins was found in VBNC, whereas dead cells do not utilise nutrients (Lleó et al., 1998). In the current study, because *L. monocytogenes* inoculated on the body is less sheltered than the calyx, there is a chance that *L. monocytogenes* might have gone into a VBNC state on the body explaining the lower recovery from this site (Highmore et al., 2018). However, further validation studies would be required.

For supply chain A, the survival of *L. monocytogenes* for both the cultivars used in the study ('Scired' and 'Cripps Pink') were not statistically different from each other ($P > 0.05$). This means that open and closed calyx cultivars used in this study did not influence the survival of *L. monocytogenes*. For inoculation regions on the apples in supply chain A, regardless of whether open or closed calyx, once *L. monocytogenes* enters the calyx (or perhaps other protected parts such as the stem-end), its survival was enhanced compared with survival on the body of apples (Nangul et al., 2021). A similar effect was observed for supply chains B and C, where 'Scired' and 'Royal Gala' represented open and closed calyx cultivars.

It is uncommon to find bacterial growth on intact surfaces as the bacteria do not have any enzymes to break the skin of the produce (MSU, 2001). In the current studies with three supply chains, *L. monocytogenes* was in a stressful environment for survival on the body of the apples. No nutrients or protection were available on the apple surface (Nangul et al., 2021). Also, gram-positive bacteria are very particular in their nutritional requirement and

unable to synthesise certain nutrients required for growth (Jay et al., 2000). Thus, although the calyx appeared to provide a more protected environment, it did not appear to provide the accessible nutrients necessary for growth (Nangul et al., 2021).

Very few studies have used dynamic temperatures to determine the fate of *L. monocytogenes* in the apple supply chain. The study by (Nangul et al., 2021) used an international supply chain, where the highest temperature used in that study was 6°C, 0.5°C being the lowest. In the current study of domestic supply chains with three different scenarios, the highest temperature observed in the three supply chains was at the grocery store's display cabinet (19-20°C) with the lowest being 0°C in the transportation truck to the DC. In the highest and lowest temperatures, although *L. monocytogenes* declined, it survived. The steps involved in the movement of fresh produce through the various markets (or supply chains) are variable, diverse, and numerous. The changing nature of produce supply chains may impact produce safety, given the many steps and the increasing distances between production and retail. Pathogens of human or animal origin may be introduced to fresh produce during production, harvest, postharvest handling, processing, storage, DC, transportation and retailing (Dallaire et al., 2006). Transportation vehicles and DCs can be constrictions within the food supply chain.

The transportation trucks that carry fresh produce from the field to the packing facility or processing plant can be a point of contamination (Warriner and Hasani, 2020), as shown in a *L. monocytogenes* outbreak in contaminated cantaloupes (Buchanan et al., 2017). The majority of the fresh produce sold in grocery stores pass through DCs and it is understood that many foods associated with foodborne illness outbreaks transit through this type of facility. Few studies previously assessed the persistence and prevalence of *L. monocytogenes* at the transportation and distribution centre (Townsend et al., 2021; Townsend et al., 2022). A study on environmental sampling of 18 DCs resulted in positive *Listeria* spp. in selected

areas of DCs (Townsend et al., 2022), and this study showed the importance of sanitation processes in *Listeria*-prone areas like floors. In the current study, temperatures that the transportation vehicles and DCs work under suggested that *L. monocytogenes* did not grow on apples. Overall, in all scenarios of the domestic supply chain, transporting from packhouse to DC, in the DC, transporting from DC to the grocery store, grocery store's cool store, and display cabinet of the grocery store, although no growth of *L. monocytogenes* was observed, the bacteria managed to survive. However, more data is required to understand bacterial persistence across different seasons multiple times. As this is the first study investigating the apple domestic supply chain, more research is needed to assess risks from this source.

3.3.3 Inactivation Kinetics of three supply chains for L. monocytogenes

Figure 3.4 shows the β and α values for the supply chains A, B and C. For inactivation kinetics, the accuracy of the Weibull model was determined by the parameter of coefficient of determination (better estimation of survival behaviour), i.e. R^2. Supply chains A and B (Table 3.3) were a good fit, with R^2 values of approximately 0.90 or higher for both. R^2 values for supply chain C were a good fit as well, with values greater than 0.90, except for 'Scired' calyx (0.785), which is still a reasonable fit for the Weibull model for inactivation of *L. monocytogenes* in apples.

Table 3.3: Weibull model showing the coefficient of determination (R^2) for every supply chain, for every cultivar at different inoculation regions.

Supply chain	Cultivar	Region	R^2
A	'Scired'	Body	0.939
	'Cripps Pink'		0.922
	'Scired'	Calyx	0.956
	'Cripps Pink'		0.873
B	'Scired'	Body	0.955
	'Royal Gala'		0.946
	'Scired'	Calyx	0.920
	'Royal Gala'		0.892
C	'Scired'	Body	0.999
	'Royal Gala'		0.981
	'Scired'	Calyx	0.785
	'Royal Gala'		0.968

Values for β (Fig. 3.4), or the shape factor for all the supply chains, although they were all significantly different ($P < 0.05$), were all $\beta < 1$, showing that weaker members of the populations are destroyed while stronger ones survived resulting in the tailing of the survival curves (Buzrul et al., 2005; Nangul et al., 2021). The ability of the organism to adapt to stress at a given time probably played some role in the tailing phenomenon as well (van Boekel, 2002).

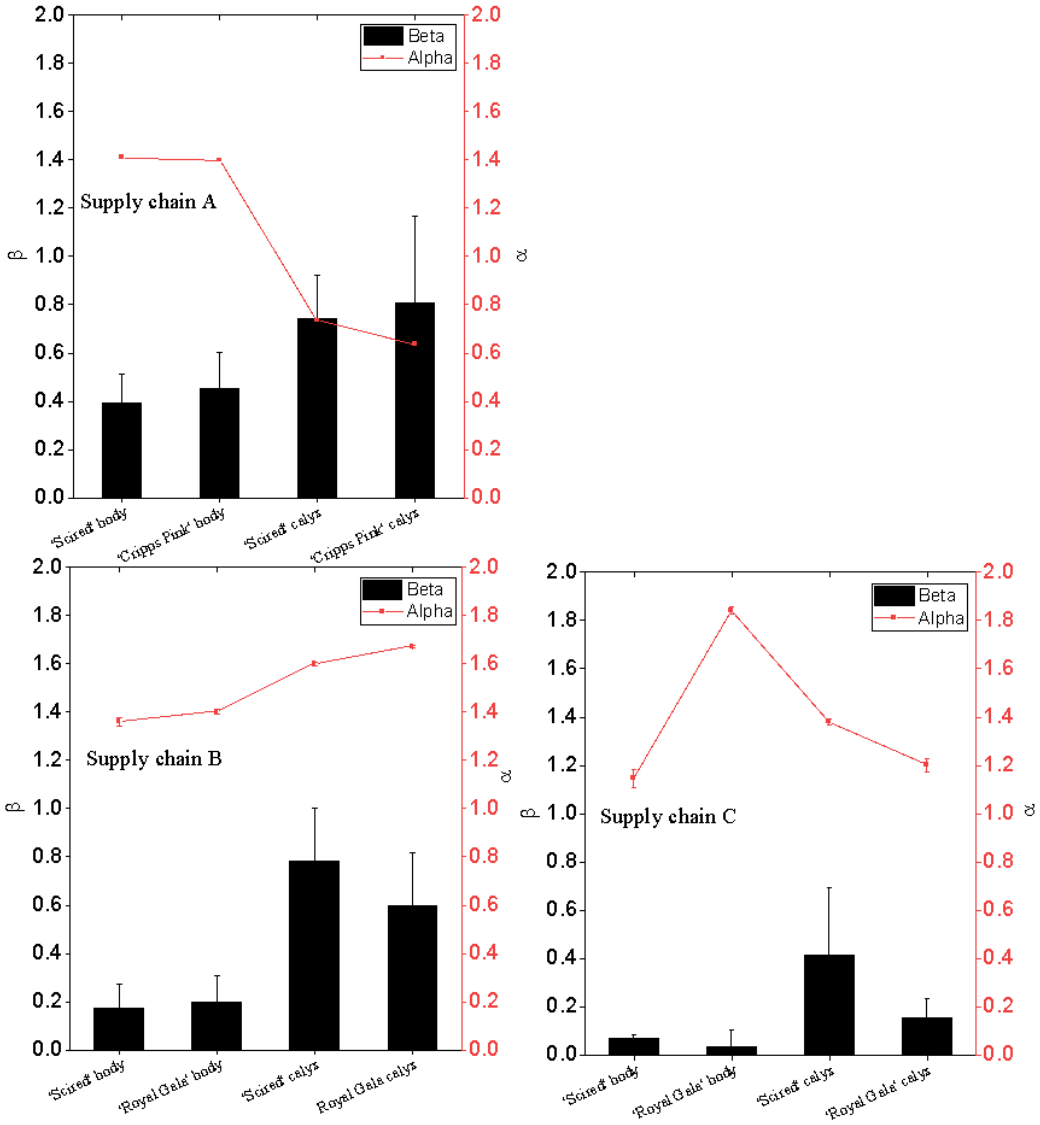


Figure 3.4: Inactivation kinetics of inoculated *Listeria monocytogenes* on the body and in the calyx, using different apple cultivars during three domestic supply chains. Weibull parameters have β and α as the shape and scale parameters, respectively. Standard deviations are represented as error bars.

β values for calyx inoculations for all the supply chain simulations were significantly higher ($P < 0.05$) than the body inoculations, which means the vulnerable populations were destroyed faster on the body than the calyx (HBM, 2021). The dynamic supply chains simulated in the current challenge studies had variable temperatures. The variable temperatures (0.5 – 20°C) in all the supply chains, which acted as a stress in the study, have affected the weaker but not the surviving cells. As a result of this, the surviving cells adapted

to the condition. The remaining survivor cells showed that the variable dynamic temperatures in the challenge studies did not mitigate the risks from *L. monocytogenes* in the domestic supply chain situations and would not be considered the only mitigation step (Nangul et al., 2021). Fifty-five case studies were studied from the literature to study the temperature dependence of two parameters; shape and scale (van Boekel, 2002). Out of 55, 14 cases had a shape factor of less than 1. Of those 14 cases, β was less than 1 for *L. monocytogenes* in cabbage juice (Beuchat et al., 1986) and milk (Chhabra et al., 1999). Also, the shape factor was < 1 for *L. innocua* in fresh-cut Chinese cabbage and *L. monocytogenes* in soybean sprouts (Alenyorege et al., 2019; Ngnitcho et al., 2018). The inactivation kinetics of other bacteria, like *Escherichia coli* and *Salmonella typhimurium*, had a shape factor < 1 in organic carrots (Liu et al., 2019). While looking into apples, $\beta < 1$ when the dynamic temperatures were simulated during the international supply chain (Nangul et al., 2021).

The change in scale factor (α) describes the effect of storage conditions on the survival of *L. monocytogenes*. Overall, at the end of each challenge study, *L. monocytogenes* on apples significantly differed ($P < 0.05$) for the dynamic temperatures in supply chains A, B and C.

For supply chain A, the value of the scale factor for inoculation on the body was higher than the calyx inoculation ($P < 0.05$), which means the body inoculations will take less time for 1 log reduction of the bacteria compared with calyx inoculations (Buzrul and Alpas, 2007). For supply chain B, calyx and body inoculations were significantly different ($P < 0.05$), with calyx inoculations higher than the body, meaning in this supply chain scenario, bacteria will die faster in the calyx than on the body. However, for the supply chain, C, α for body inoculation were different to the calyx ($P < 0.05$), except for the 'Scired' body and 'Royal Gala' calyx, which is a mixed response to supply chain A and B. More data and challenge studies are needed to describe the scale factor and different supply chain scenarios for *L. monocytogenes* in apples.

For supply chain A, the scale factor (α) after the conclusion of the challenge study was 0.64 and 0.72 for 'Cripps Pink' and 'Scired' calyx ($P < 0.05$), and 1.4 and 1.4 for 'Cripps Pink' and 'Scired' body ($P > 0.05$). This outcome means that cultivars play a role in reducing the concentration of *L. monocytogenes* in the calyx for 'Scired' compared with 'Cripps Pink' for supply chain A. For supply chain B, α for 'Scired' and 'Royal Gala' calyx was 1.6 and 1.67 ($P > 0.05$), respectively, and 1.37 and 1.40 for 'Scired' and 'Royal Gala' body ($P > 0.05$), respectively. No effect of cultivars for supply chain B in the calyx or on the body was observed. For supply chain C, α for 'Scired' and 'Royal Gala' calyx was 1.40 and 1.24 ($P < 0.05$), respectively, and 1.17 and 1.80 for 'Scired' and 'Royal Gala' ($P < 0.05$), respectively. No previous literature was available on the apple cultivar effects in the domestic supply chain scenarios, which would be needed to draw further conclusions.

3.4 Conclusion

The study demonstrated the effect of the domestic supply chain temperatures on *L. monocytogenes*, on the body and calyx of various apple cultivars, including trucking, DCs, and retail stores. *L. monocytogenes* is known to survive and grow at temperatures ranging from 0 – 40°C; however, in apple cultivars used in this study, no bacterial growth was observed. This was the first study on the variable temperatures on which trucking, DCs, retail stores operate, in which the bacteria did not grow. However, more data is needed to confirm the outcome. The results presented in the current study (i.e. no growth of *L. monocytogenes* in apple regions) could be beneficial for the postharvest supply chain handlers for the temperatures they work under. However, further microbial testing along the supply chain and other studies are recommended to ensure risk minimisation. To successfully minimise bacterial pathogens, it is critical to control the temperature at every step of postharvest handling (Beuchat and Ryu, 1997). Otherwise, opportunistic bacteria like *L. monocytogenes* will persist.

3.5 Funding Source

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Preface to Chapter 4

In Chapters 2 and 3, both the international and domestic supply chains were studied. A cocktail of three lineages of *L. monocytogenes* was used in both supply chains. As every lineage of *L. monocytogenes* is pathogenic, it essential to understand which lineage survives better, especially at the temperatures at which the apple supply chain works. The reason is that if the particular lineage is known, a targeted mitigation strategy can be applied in case of an outbreak.

It is important to note that *L. monocytogenes* is widely distributed in the environment and linked to major outbreaks. A critical step toward controlling and preventing listeriosis outbreak is to detect and identify *L. monocytogenes* in any fresh produce as rapidly as possible. This chapter will compare rapid detection techniques like qPCR with conventional techniques like the MPN method. To understand the bacterial growth potential, the chapter will also investigate the high and lower inoculum of each lineage of *L. monocytogenes* on the apple body and calyx in storage.

Chapter 4: The effect of storage temperatures on the survival of three lineages of *Listeria monocytogenes* on apples

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Abstract:

Listeria monocytogenes (*L. monocytogenes*) isolates can be grouped in four lineages. Lineage I is a principal causative agent of clinical listeriosis, while lineage II is frequently found in food and processing environments. Lineages III and IV are rarely isolated. To understand the effect of temperature on *L. monocytogenes* and its lineages, two storage studies were performed under static temperature conditions on the 'Scired' apple cultivar. (i) A seven-strain cocktail (10^8 cells – high inoculum and 10^6 cells – lower inoculum) of three lineages of *L. monocytogenes* was inoculated onto the body and into the calyx of apples that were then stored at 0.5, 2, 6 and 20°C for two weeks; and assessed using most probable number (MPN). (ii) Effects of individual lineages I, II, and III were investigated with high (10^8 cells) and lower (10^6 cells) inocula using MPN and qPCR methodologies. Both studies sampled apples after days 1, 4, 7, 10, and 14 and quantitatively assessed *L. monocytogenes*. Both studies observed no growth for the high or lower bacterial inocula. Regardless of inoculum concentration, the bacteria survived significantly better ($P < 0.05$) in the calyx than on the body. At all the temperatures used in this study, *L. monocytogenes* concentrations differed

significantly ($P < 0.05$). The qPCR study showed that lineage III declined more rapidly than lineage I and II on the body and calyx for both high and low inoculations. However, the higher bacterial concentrations detected by the qPCR could be due to nucleic acids originating from dead cells interfering with the final results. The study gave good insights into measuring individual *L. monocytogenes* lineages for apples and the importance of rapid detection methodologies.

Keywords: Inoculation region, inocula size, qPCR, lineage

4.1 Introduction

Listeriosis is an infection caused by a gram-positive facultative intracellular pathogen named *Listeria monocytogenes (L. monocytogenes).* According to the World Health Organisation (WHO), approximately 600 million people worldwide contract listeriosis each year, leading to 420,000 deaths (Bhunia, 2018). *L. monocytogenes* is a ubiquitous opportunistic bacterial pathogen that can contaminate ready-to-eat (RTE) fruit such as apples. Approximately 99% of *L. monocytogenes* infections are foodborne (Swaminathan and Gerner-Smidt, 2007). *L. monocytogenes* can be transferred to patients from ready-to-eat tree fruits such as stone fruit and apples (Angelo et al., 2017; Buchanan et al., 2017; Jackson et al., 2015; Nangul et al., 2021). *L. monocytogenes* outbreak related to apples in 2014 highlighted severe public health and economic issues related to food process contamination and foodborne illness (Ruiz-Llacsahuanga et al., 2021a).

Apples are an essential commercial fruit crop for several countries worldwide, including New Zealand, and account for almost a quarter of the export value of fresh produce (MBIE, 2017). Contamination of apples with *L. monocytogenes* and subsequent fruit recalls (FDA, Food and

Drug Administration 2019) highlighted the importance of investigating new measures to mitigate the risks of foodborne pathogens in food processing systems.

Due to the high mortality in cases of listeriosis, governments and food safety agencies worldwide are taking serious steps to reduce *L. monocytogenes* in the food production chain (Orsi, Bakker, et al., 2011). For RTE products, the U.S. Department of Agriculture's Food Safety and Inspection Service established a debatable "zero tolerance" policy, making the food industry difficult to achieve compliance (Bhunia, 2018). Due to this "zero tolerance," the annual estimated cost of food recalls related to *L. monocytogenes* in the U. S. ranges between $1.2 - $2.4 billion (Ivanek et al., 2004).

It is agreed that *L. monocytogenes* is a challenging bacterium due to its psychrotolerant and its ability to grow at temperatures from -1.5 to 45°C, high salt concentrations of up to 10% and pH levels between 4.0 - 9.6 in a variety of foods (Angelo et al., 2017; Bucur et al., 2018; Chan and Wiedmann, 2008; Marik et al., 2020; Mcclure et al., 1989; Nangul et al., 2021; Sheng et al., 2017). *L. monocytogenes* have four serotype-associated lineages: I, II, III and IV. Different lineages have different characteristics, particularly concerning pathogenic potential (Doijad, Weigel, et al., 2015; Rawool et al., 2016). Lineage I (LI) isolates include major epidemic clones of *L. monocytogenes* associated with human listeriosis cases (Sauders et al., 2006). Lineage II (LII) isolates are most commonly isolated from foods and the environment (Kathariou, 2002; Mohan et al., 2021). LI, as a result of its genetic variation, is more infectious to humans than LII (Bechtel and Gibbons, 2021; Nightingale et al., 2005), with LII showing reduced virulence due to the presence of a premature stop codon in over 30% of LII isolates (Nightingale et al., 2005). Lineage III (LIII) is less common and mostly found in environmental samples, while lineage IV isolates are rare and primarily found in animal hosts (Kathariou, 2002; Orsi, den Bakker, et al., 2011; Weidmann, 2020). Few methodologies are available to detect and identify the individual lineages of *L.*

monocytogenes or the bacteria in general. A recent study using dynamic temperature conditions showed that a cocktail of *L. monocytogenes* comprising three different lineages on apples could survive but did not grow under the variable temperatures experienced during conditions of sea-freighting from New Zealand (Nangul et al., 2021). Furthermore, *L. monocytogenes* numbers on apples declined considerably in the first two weeks of the 12 and 20-week shipping simulation periods. However, it was unclear whether the reduction of bacteria resulted from the dynamic condition. Few studies have used static temperatures to study the survival of *L. monocytogenes* on apples, and the study outcomes contradict each other (Macarisin et al., 2019; Sheng et al., 2017). As a result, more studies using static temperatures to study the *L. monocytogenes* by observing the survival are warranted.

It is important to note that the previous study (Nangul et al., 2021) was carried out at the high inoculum concentration (10^8 colony forming units – CFU/mL), and the study on low inoculum concentration was not part of that experiment. An inoculum concentration is considered high when it gives a moderate to maximum infectious dose response in animal infection experiments. However, it involves various probabilities (Van Stelten et al., 2011). Although no epidemiological data described the minimum or maximum inoculum concentrations to describe the infectious dose, it is estimated that 10^6 colony-forming units (CFUs) in immunocompromised individuals to 10^9 CFUs in healthy individuals is the estimated dose to get infected, respectively (Quereda et al., 2021). In the current study by (Nangul et al., 2021), a high inoculum concentration could be too much to give realistic results for *L. monocytogenes*, especially on an intact fruit such as an apple. Recent trials identified that inoculation concentrations affect the outcomes of such studies, and lower initial inoculation concentrations may lead to remarkable growth potential (McManamon et al., 2017). The growth potential of *L. monocytogenes* is the ability of the bacteria to grow under certain conditions. According to the European Union Reference Laboratory (EURL),

any food product is permissive to the growth of *L. monocytogenes*, if it has a growth potential greater than 0.50 $\log_{10}$ CFU/g (Culliney and Schmalenberger, 2020). Despite the possible underestimation of growth potential, assessing growth potentials at high inoculation densities in RTE food remains popular (Ziegler et al., 2019). Previous results suggested that the fate of large concentrations of inoculated pathogens may or may not mirror natural contamination loads (Flessa et al., 2005). Notably, when apples were inoculated at high and low inoculum levels, the concentration of low inoculum slightly increased in the first 24 h but remained constant for two weeks (Sheng et al., 2017). So, the capacity of the bacteria at lower inoculum levels should be investigated in food systems and compared with high inocula to understand the bacterial growth potential and carrying capacity (the maximum population of bacteria that its environment can maintain). However, previous studies have already suggested that the bacterial growth potential of *L. monocytogenes* differs amongst serotypes (Norton et al., 2001), and the methods to detect them take considerable time.

The most commonly used method for detecting and identifying *L. monocytogenes* in foods worldwide are conventional culture method using selective and chromogenic media (Kim et al., 2014). Due to the major *L. monocytogenes* outbreaks worldwide, it is critical to quickly identify, prevent, and control the outbreak (Chen et al., 2017). However, the major disadvantage of selective and chromogenic media is the time to get the positive result after sample collection, 5-7 days (Norton, 2002). There is a need to examine the methodologies of identifying *L. monocytogenes* lineages through conventional methods and molecular techniques (Kim et al., 2014). Also, molecular techniques like real-time quantitative PCR (qPCR) are an effective methodology for food samples and complement standard culture methodologies (Churchill et al., 2006; Jantzen et al., 2006; Kim et al., 2014). The qPCR has become helpful for detecting and quantifying microorganisms as it is highly sensitive and accurate. This method involves amplification cycles in which DNA is denatured and annealed

with primers, leading to an exponential increase of amplicons monitored in real-time at every cycle with a fluorescent dye (Chen et al., 2017). We thus concluded from the literature that the potential of the three main *L. monocytogenes* lineages (lineage I, II and III) to grow on apples should be individually studied using conventional and molecular techniques.

In the present study, we aimed to investigate the effect of *L. monocytogenes* at different static temperatures on a New Zealand-grown apple cultivar using high and low inoculation concentrations and on different lineages. The aim was achieved using two trials by studying the:

A. Effect of different static temperatures on high and lower inocula of a *L. monocytogenes* cocktail evaluated using a most probable number (MPN) method.

B. Effect of different static temperatures on high and lower inocula of individual *L. monocytogenes* lineages evaluated using the MPN and qPCR method.

4.2 Materials and methods

4.2.1 Apples

Apples (*Malus domesticus*) were obtained from a commercial packhouse in Hawke's Bay, New Zealand. The apples were of an open calyx cultivar, 'Scired', with 100 count size for an 18 kg carton. Apples were un-waxed and of export quality with no bruising, cuts, or scars. The fruit had been through conventional packhouse treatments like the use of sanitisers.

4.2.2 Listeria monocytogenes strains

To account for variation in growth and survival rates amongst bacterial strains, six genetically different *L. monocytogenes* isolates of different lineages isolated from New Zealand horticultural sources (PFR46G06, PFR46E10, PFR40I07, PFR41I04, PFR41F08, PFR41H05), and one clinical isolate (Scott A) (Plant & Food Research, Auckland, New Zealand) were chosen. No lineage IV isolates from horticultural sources were available to

include in the study. The explanation of every lineage and its sequence type is already described in previous chapters (Nangul et al., 2021).

4.2.3 Challenge study set-up

The research aim was investigated using the following trial studies:

Trial A: In this study, seven isolates from three lineages were chosen to make the high and lower inoculum bacterial cocktails, and the effect of static temperatures of 0.5, 2, 6 and 20°C for 14 days on *L. monocytogenes* was determined.

Trial B: In this study, the effect of static temperatures on single lineage cocktails of the three lineages of *L. monocytogenes* was determined using the MPN and real-time qPCR method.

4.2.4 Inoculum preparation

L. monocytogenes strains were assessed for purity by streaking them on tryptic soy agar with 0.6% added yeast extract (TSAYE) (Becton, Dickinson & Company, USA) and then onto *Listeria* CHROMagar™ plates (CHROMagar™, Paris, France). The resulting colony morphologies were assessed for appearance and uniformity. Pure bacterial cultures were then grown in TSBYE for 48 h at 37°C to achieve a stationary growth phase. The strains were centrifuged at 4000 rpm (3220g) (model 1736R, Gyrozen, Seoul, South Korea) for 10 min at 4°C. The resulting pellets were washed twice with 0.1% peptone (10 mL) (Bacto™, BD Biosciences, USA).

For trial A, equal volumes of all seven *L. monocytogenes* strains were combined to make a seven-strain cocktail with final concentrations of 10^8 CFU/mL (high inoculum) and 10^6 CFU/mL (lower inoculum), which were used for apple inoculation. For trial B, cocktails of equal volumes of two (LII and LIII) or three strains (LI) were independently made with final concentrations of 10^8 colony-forming units (CFU/mL) (high inocula), and 10^6 CFU/mL (lower inocula) and these single lineage cocktails were used for apple inoculation. The reason

for using 10^6 as the lower and 10^8 CFU as the high inoculum is that this is about the minimum and maximum infectious dosage required to get immunocompromised and healthy individuals sick (Quereda et al., 2021).

4.2.5 Inoculation of apples

The procedures to inoculate and store the apples were kept the same for all the trials. Whole unwaxed apples were removed from cold storage (0.5°C) and left at room temperature (20°C) overnight before inoculation. The following day, apples were inoculated with the high or lower inocula of the relevant *L. monocytogenes* cocktail. For each cultivar, apples were divided into two groups: (i) calyx inoculation - 50 µL of *L. monocytogenes* cocktail was pipetted into the calyx of each apple and (ii) body inoculation - two droplets of 25 µL each were pipetted onto the equatorial region. The apples were air-dried for three hours in a class II biosafety cabinet at room temperature until visibly dry.

4.2.6 Apple storage and sampling

After air drying, apples were stored in temperature-controlled rooms at 0.5, 2, 6 and 20°C. Temperature monitoring of the rooms were logged at 1 min intervals using type-T thermocouples and a Grant Squirrel 1000 Series meter/logger (Type 1025; Grant Instruments Ltd, Barrington, Cambridge, UK).

Sampling was carried out on days 0 (after air drying), 1, 4, 7, 10 and 14. At each storage time-point, nine fruit (three sets of three apples) were sampled for each inoculation type, and *L. monocytogenes* populations were determined below.

4.2.7 Bacterial enumeration from apples

Three sets of three apples were quantitatively assessed for *L. monocytogenes* by sampling the body and the calyx using a most probable number (MPN) method to detect stressed organisms (Osborne and Bremer, 2002).

For the body, each set of apples was placed into a sterile bag with 400 mL of Buffered *Listeria* Enrichment Broth (BLEB) (Acumedia, Lansing, Michigan, USA) and hand massaged for two min. Wash solution, 2 mL from each bag, was then aliquoted into triplicate 15 mL Falcon tubes. Aliquots from each tube were subjected to ten-fold serial dilution in BLEB (200 µL) in 96 well micro-titre plates. Undiluted and diluted samples were then enriched by incubating for 48 h at 30°C. After 48 h, for trials A and B, 2 µL solutions from each bag, tubes and micro-titre wells were spotted onto pre-gridded selective CHROMagar™ *Listeria* plates, which were incubated for 48 h at 37°C to determine *L. monocytogenes* presence or absence. Positive readings of *L. monocytogenes* for the MPN tubes or wells were identified by recording blue cultures on the CHROMAgar. For the qPCR method, the protocol used was the same until the enrichment for 48 h at 30°C. After enrichment, 2 ml aliquots were used to extract DNA for qPCR.

For the enumeration from calyx, the core of each apple was taken out with a sterile cork-borer, weighed, and homogenised in a laboratory stomacher (Smasher, AES Chemunex, AES laboratory, France) for two minutes in BLEB (1:10). This was followed by the same protocol used for enumeration on the body inoculation for trials A, B. The MPN values were calculated using the Bacteriological Analytical Manual (BAM) spreadsheet (Blodgett 2010). All results were expressed as $\log_{10}$ MPN per apple with a detection limit of 1.37 $\log_{10}$ MPN/ apple based on presence/absence results from the tubes and wells and the three apples from the bags.

4.2.8 Genomic DNA extraction and real-time qPCR quantification

For Trial B (qPCR method), to quantify the number of *L. monocytogenes* present during the storage period, genomic DNA from the high and lower inocula was extracted from a subsample from each bag, tube and well after the enrichment process (30°C in BLEB for 48 h). The subsample was taken for the DNA extraction so that the results could be directly

compared with the MPN enrichments. The genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen, Melbourne, Australia) following the manufacturer's instructions. The PCR primers used in this study were from a pre-published source (Rawool et al., 2016).

Real-time PCR quantification of *L. monocytogenes* was performed using a LightCycler 480 instrument (Roche Diagnostics, Mannheim, Germany). The PCR reactions were carried out in duplicate with a reaction volume of 10 µL, consisting of 5 µL of LightCycler 480 SYBR Green I Master mix, 0.5 µM of each primer and 2 µL of DNA template and with 3 µL sterile water.

The qPCR cycling condition for lineages I and II included an initial denaturation of DNA at 95°C for 5 mins, followed by 55 cycles at 95°C for 15 s, 60°C for 30s, and 72°C for 20s. The cycling condition of the qPCR for lineage III included an initial denaturation of DNA at 95°C for 5 mins, followed by 55 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 12 s. The qPCR results (CFU/ml) were converted to $\log_{10}$ MPN/apple to compare directly with the MPN method.

4.2.9 Statistical analysis

Microbial data for trials A and B (MPN and qPCR) were analysed using GenStat (version 18th; VSN International Ltd). The data was analysed using analysis of variance (ANOVA) followed by post-hoc Tukey's test. In trials A and B, results were presented as means of the log values with standard deviations in the graphs, with high and lower inoculum microbial data regarded as a variate. A value of $P < 0.05$ was considered statistically significant. All the figures were prepared using the Origin software 2021b (OriginLab Corporation, Northampton, USA).

4.3 Results and discussion

4.3.1 Trial A: Effect of static temperatures on a seven-strain L. monocytogenes cocktail

As mentioned before, the initial titres of the 50 µL *L. monocytogenes* inocula applied to the body and calyx of apples were 10^8 CFU/mL for the high and 10^6 CFU/mL for the lower inoculum.

Four hours after inoculation, the mean recovered titres in the calyces were 2.4 x 10^7 and 3.3 x 10^5 MPN/apple for the high and lower inocula, respectively, and on the body, were 1.9 x 10^6 and 6.5 x 10^4 MPN/apple for the high and lower inocula, respectively. The drop in *L. monocytogenes* titre on the body could be due to the drying process causing cell damage, but in the more enclosed environment of the calyx, drying might be slower, resulting in less damage.

At all temperatures, *L. monocytogenes* showed no growth for high or lower inocula, either in the calyx or the body (Figure 4.1 (A and B). As shown in Figure 4.1A, for the high inoculum, apples stored at 0.5, 2, 6, and 20°C respectively, gave final *L. monocytogenes* concentrations in the calyx of 5.88, 6.66, 5.32, and 4.82 $\log_{10}$ MPN/apple corresponding to reductions of 1.50, 1.32, 2.06, and 2.56 $\log_{10}$ MPN/apple respectively. On the body, the high inoculum resulted in log reductions of 2.69, 3.47, 3.82, and 3.41 $\log_{10}$ MPN/apple, respectively. For the lower inoculum in the calyx, temperatures of 0.5, 2, 6, and 20°C resulted in log reductions of 2.22, 1.62, 2.05, 2.56 $\log_{10}$ MPN/apple, respectively, after the 14 days storage period (Figure 4.1B). After just seven days at 0.5, 2, 6, or 20°C, the lower inocula on the body were below the detection limit (<1.37 $\log_{10}$ MPN/apple), giving a log reduction of greater than 4.68 $\log_{10}$ MPN/apple regardless of storage temperatures, respectively.

Regardless of the temperatures and inoculum sizes, *L. monocytogenes* survived significantly better in the calyx than on the body (P <0.05). This might be due to the unavailability of nutrients or bodily protection. The bacteria may be more protected and hidden in the crevices

in the calyx, which could act as a harbour site for bacterial attachment (Nangul et al., 2021; Pietrysiak and Ganjyal, 2018). The apple cores were homogenised for sampling in the calyx, which meant that some of the acidic apple flesh was included. This, if anything, might be expected to reduce numbers compared to those found on the body, but the opposite was found. Similar results were noted previously by Nangul et al. (2021) and in a review by (Marik et al., 2020).

It was expected to find growth on the lower inoculums, either in the calyx or on the body of the apple. However, the current study observed no growth at either of the inoculum levels. Previous studies suggested that compared to higher inocula, lower inoculum concentrations have been reported to provide more *L. monocytogenes* growth potential due to carrying capacity (the maximum population of bacteria that its environment can maintain) (Marik et al., 2020). Lower inoculum concentrations may help explain *L. monocytogenes* growth potential, but that depends on the carrying capacity of the inoculated produce (Marik et al., 2020). For example, when strawberry samples were inoculated at higher (7.5 $\log_{10}$ CFU/berry) and lower (5.6 $\log_{10}$ CFU/berry) inoculation concentrations at ambient temperatures, *L. monocytogenes* concentrations declined much faster in the lower inoculum than in the high inoculum (Flessa et al., 2005).

On the other hand, *L. monocytogenes* populations inoculated onto mangoes at low and high inoculum concentrations (3 and 6 log CFU/mango, respectively) grew to reach similar maximum concentrations (about 6.5 log CFU/mango) (Danyluk, 2017). The carrying capacity of different types of fresh produce depends on factors such as background microflora and surface properties (Buchanan and Bagi, 1997; Marik et al., 2020). For example, research of parsley inoculated with 10^3, 10^7 and 10^8 CFU/leaf determined the carrying capacity of parsley to be 10^5 CFU/leaf (Dreux et al., 2007). The carrying capacity of apples has not been established. However, carrying capacity does not appear to have influenced the results

presented in Figure 4.1. Rather than growing towards the carrying capacity, at least on the body of the apples, the lower inoculum *L. monocytogenes* declined more than the high inoculum. The result suggested that the carrying capacity of apples (in the calyx or body) could be lower than 6 $\log_{10}$ MPN/apple. However, as this is the first research done on apples, more research and data are needed to verify the results.

Bacteria on the body of an apple is not expected to grow. The growth on intact surfaces like the apple's body is unlikely as bacteria do not possess any enzymes to break the fruit's skin (Michigan State University (MSU, 2001)). For both high and low inocula in the current study, *L. monocytogenes* on the skin of the apple would be in a stressful environment for survival. No nutrients or protection were available on the apple surface, whereas in the calyx, the bacteria may be protected and hidden in microstructures such as cracks and crevices of the apple. These microstructures in the calyx may serve as a harbour site for bacterial attachment by physical entrapment (Nangul et al., 2021; Pietrysiak and Ganjyal, 2018). Also, gram-positive bacteria (like *L. monocytogenes*) are generally specific in their nutritional requirement and cannot synthesise certain nutrients required for growth (Jay et al., 2000). Thus, although the calyx appeared to provide a more protected environment, it did not appear to offer the accessible nutrients necessary for growth (Nangul et al., 2021).

It is important to note that the apple skin thickness varies considerably between cultivars and between different years, even within the same cultivar. Generally, the skin is thicker at the stem end of fruits than at the stylar end, whereas the thickness is intermediate at the point of maximum fruit diameter. The cuticle is also thicker on the shaded than on the sun-exposed side of apple. The skin characteristics influence fruit bruising to a large extent. The skin thickness influences Ca penetration into apple fruit and also the penetration of fungal pathogens into fruits (Homutová, I. & Blažek, J., 2006).

In the current study, regardless of inoculation on the body or in the calyx, the rate of decline of lower *L. monocytogenes* inoculum was more rapid than higher inoculum. This result was similar to the research on strawberries, where *L. monocytogenes* declined significantly in the lower inoculum (decline of 3.3 log CFU/berry) than in the high inoculum (decline of 1.4 log CFU/berry). Like *L. monocytogenes*, *Shigella* also declined more rapidly at lower (5.0 log CFU/berry) than higher (7.0 log CFU/berry) inocula (Flessa and Harris 2002). The reason for this remains unknown. However, we hypothesise that compared to the lower inoculum, the surviving higher inoculum bacteria are nourished and protected by the large pool of already dead bacteria in their microenvironment, resulting in the high inoculum bacteria surviving better than the lower inoculum. Also, the above study for high and lower inoculum was done at different storage temperatures, which is essential to discuss bacterial survival.

At the end of the study, the survival of *L. monocytogenes* in the calyx tended to survive more at lower temperatures than at higher temperatures. It has already been verified that temperature is critical in *L. monocytogenes* growth (Nangul et al., 2021). In the current study, the effect of different temperatures on *L. monocytogenes* in both high and lower inocula was significantly different ($P < 0.05$), which means *L. monocytogenes* survival changes at different temperatures. At low temperatures, the *L. monocytogenes* bacterial membrane becomes rigid, and its metabolic rate decreases. At the genetic and protein level, *L. monocytogenes* possess different mechanisms that trigger under low-temperature conditions. For example:

- The bacterium increases its expression of the genes required in cell membrane function, producing cold shock proteins and several molecular schemes to sustain homeostasis or maintain its relatively stable internal state (Santos et al., 2019).
- The survival of the bacteria at the low temperatures results in the bacteria going under stressful conditions, which increases the amount of osmolyte and peptide transporters in the cytosol to help maintain the turgor pressure in *L. monocytogenes* (Miladi et al., 2017).

- Also, bacterial protein damage due to protein misfolding can occur under cold environmental conditions. To counteract this damage, cells have molecular chaperons (any protein that interacts with and aids in the folding or assembly of another protein without being part of its final structure) that help maintain the proteins in their original state (Kim et al., 2013; Santos et al., 2019).
- Upon exposure to low temperatures, cold shock proteins (CSPs) and cold acclimation proteins (CAPs) are induced at different levels (Chan and Wiedmann, 2008). For CAPs, *L. monocytogenes* possesses three proteins from the family of CspA (CspLA, CspLB, and CspD), whose expression increases at low temperatures (Chan and Wiedmann, 2008).

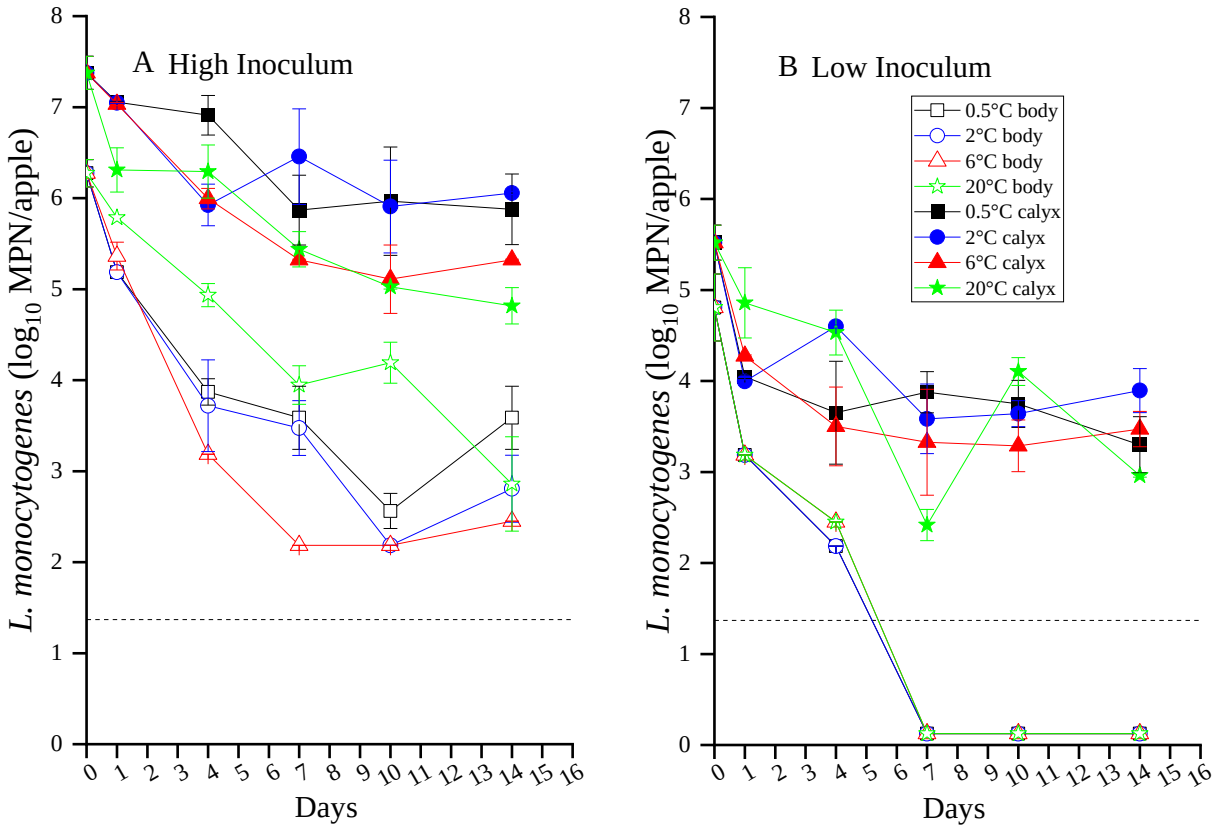

Figure 4.1: Survival of inoculated *L. monocytogenes* on the body and in the calyx of the apples under four different static temperature scenarios: 0.5, 2, 6, and 20°C for 14 days. Figures 4.1 (A) and (B) show high and low inoculum survival. The dotted horizontal line shows the 1.37 $\log_{10}$ MPN/apple detection limit.

The above study suggested the fate of *L. monocytogenes* when all the lineages were present in the cocktail at high and low inoculum. However, as every lineage of *L. monocytogenes* has a unique genetic make-up (Orsi, Bakker, et al., 2011), it is unclear if each lineage behaves the same way as they are present in a cocktail. Trial B investigated the fate of each lineage of *L. monocytogenes* (from three lineage strains used in the cocktail for trial A), for high and lower inocula at different temperatures and compared the bacterial enumeration using MPN and qPCR methods.

4.3.2 Trial B: Effect of static temperatures on three individual lineages of L. monocytogenes applied at high and lower inocula and evaluated using the MPN and qPCR method

4.3.2a MPN method

The survival of *L. monocytogenes* was studied for individual lineages using MPN and qPCR, applying them separately using high and lower inocula (Figures 4.2, 4.3 for MPN, respectively and Figures 4.4, 4.5 for qPCR, respectively).

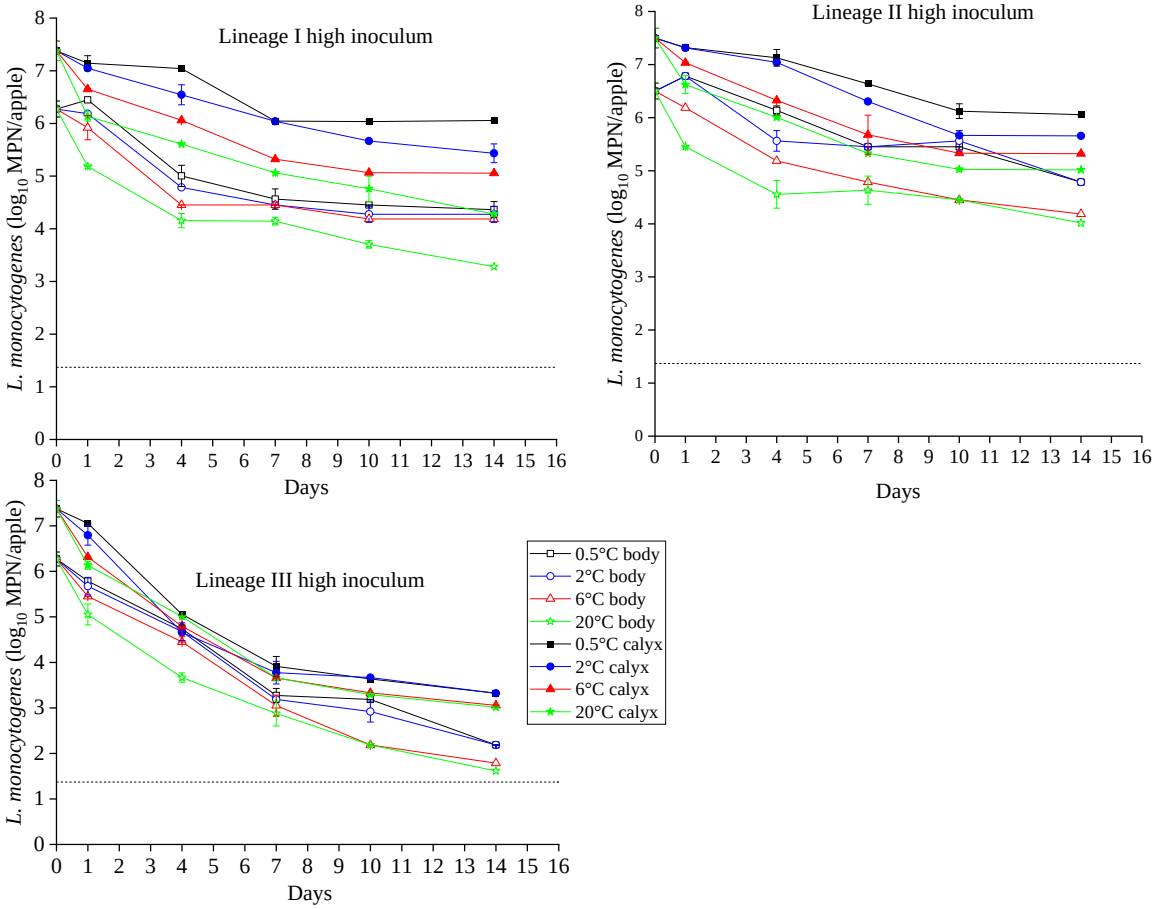

Figure 4.2: Survival of high inocula individual cocktails of *L. monocytogenes* lineages I, II, and III inoculated on the body and in the calyx of the apples under four different static temperature scenarios: 0.5, 2, 6, and 20°C for 14 days. The dotted horizontal line shows the 1.37 $\log_{10}$ MPN/apple detection limit

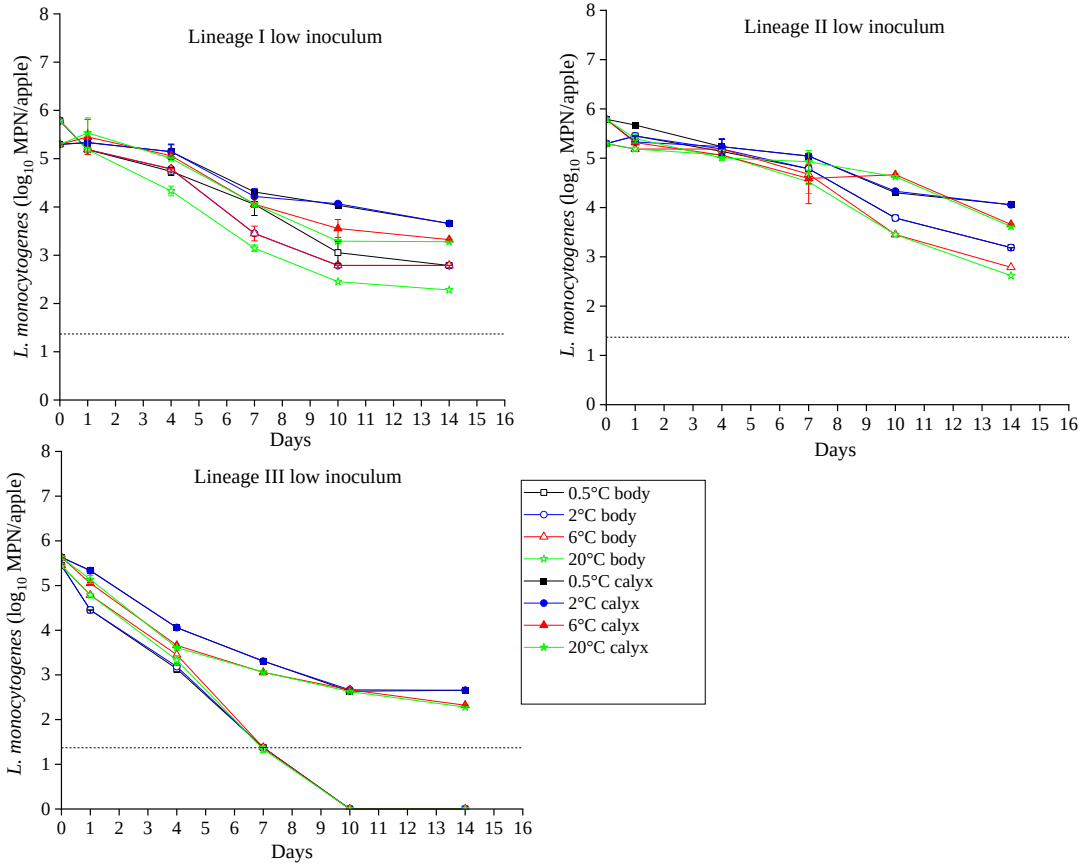

Figure 4.3: Survival of lower inocula individual cocktails of *L. monocytogenes* lineages I, II, and III inoculated on the body and in the calyx of the apples under four different static temperature scenarios: 0.5, 2, 6, and 20°C for 14 days. The dotted horizontal line shows the 1.37 $\log_{10}$ MPN/apple detection limit.

Table 4.1 below shows the concentrations of the different lineages of *L. monocytogenes* on apples at the start and end of 14 days of storage.

Table 4.1: Concentrations of different lineages of high and lower inoculum of *Listeria monocytogenes* on apples at the start and end of 14 days of storage at 0.5, 2, 6, and 20°C. Each temperature was analysed individually. Data with the same letters in the parenthesis of each temperature are not significantly different (P > 0.05), while those with different letters are different (P < 0.05) from each other. Column heading with 'Lin.' denotes lineages of *L. monocytogenes*. Column heading with 'Inoc. region' should be read as 'Inoculation region', whereas 'Inoc. size' is 'Inoculation size'.

Lin.	Inoc. region	Inoc. size	(log_{10} MPN/apple)											
			0.5°C			2°C			6°C			20°C		
			Initial	Final	Red.	Initial	Final	Red.	Initial	Final	Red.	Initial	Final	Red.
I	Calyx	High	7.38^{n}	6.06^{lm}	1.32	7.38^{l}	5.43^{hi}	1.95	7.38^{n}	5.06^{i}	2.32	7.38^{o}	4.28^{i}	3.1
		Lower	5.30^{i}	3.66^{e}	1.64	5.30^{h}	3.66^{e}	1.64	5.30^{ij}	3.32^{f}	1.98	5.30^{k}	3.28^{f}	2.02
II		High	7.38^{n}	6.06^{lm}	1.32	7.38^{l}	5.66^{ij}	1.72	7.38^{n}	5.32^{j}	2.06	7.38^{o}	5.02^{j}	2.36
		Lower	5.30^{i}	4.06^{f}	1.24	5.30^{h}	4.06^{f}	1.24	5.30^{ij}	3.66^{g}	1.64	5.30^{k}	3.62^{g}	1.68
III		High	7.38^{n}	3.32^{d}	4.06	7.38^{l}	3.32^{d}	4.06	7.38^{n}	3.06^{e}	4.32	7.38^{o}	3.02^{e}	4.36
		Lower	5.63^{jk}	2.66^{c}	2.97	5.63^{ij}	2.66^{c}	2.97	5.63^{kl}	2.32^{c}	3.31	5.63^{lm}	2.28^{c}	3.35
I	Body	High	6.27^{m}	4.36^{g}	1.91	6.27^{k}	4.27^{f}	2	6.27^{m}	4.19^{h}	2.08	6.27^{n}	3.28^{f}	2.99
		Lower	5.79^{kl}	2.79^{c}	3	5.79^{j}	2.79^{c}	3	5.79^{l}	2.45^{c}	3.34	5.79^{m}	2.28^{c}	3.51
II		High	6.27^{m}	4.79^{h}	1.48	6.27^{k}	4.79^{g}	1.48	6.27^{m}	4.19^{h}	2.08	6.27^{n}	4.02^{h}	2.25
		Lower	5.79^{kl}	3.19^{d}	2.6	5.79^{j}	3.19^{d}	2.6	5.79^{l}	2.79^{d}	3	5.79^{m}	2.62^{d}	3.17
III		High	6.27^{m}	2.19^{b}	4.08	6.27^{k}	2.19^{b}	4.08	6.27^{m}	1.79^{b}	4.48	6.27^{n}	1.62^{b}	4.65
		Lower	5.45^{ij}	$<1.37^{a}$	>4.08	5.45^{hi}	$<1.37^{a}$	>4.08	5.45^{jk}	$<1.37^{a}$	>4.08	5.45^{kl}	$<1.37^{a}$	>4.08

For the high inoculum, all the lineages under all the temperatures, *L. monocytogenes* survived better in the calyx than on the body ($P < 0.05$), which followed the same trend as in Figure 4.1 as discussed above. Lineages I and II survived better ($P < 0.05$) than lineage III at both high and lower inocula, either in the calyx or on the body. Literature suggests that on average, lineage I are more virulent than lineage II with strains from lineage I being more commonly linked to outbreaks involving human cases (Pirone-Davies et al., 2018). However, lineage II isolates in food-related environments are more prevalent than lineage I isolates (Chen et al., 2009; Gianfranceschi et al., 2003; Orsi, Bakker, et al., 2011; Sauders et al., 2004). The reason behind the moderately increased prevalence of lineage II over lineage I, especially at high temperatures (6 and 20°C in this case, a usual food processing facility temperature) may be the ability of lineage II to survive in food-associated environments, sometimes even up to 12 years (Orsi et al., 2008). Also, lineage II seems more resistant to bacteriocins than lineage I (Buncic et al., 2001), which could provide a mild selective advantage for lineage II in food samples containing bacteriocin-producing organisms (Orsi, Bakker, et al., 2011). There are quite a few differences between lineage I and lineage II strains. For example, lineage II strains display higher recombination rates than lineage I, leading to an enhanced capacity to adapt to various environments (Pirone-Davies et al., 2018). This might explain why lineage II strains are predominantly found in the food and food environments than lineage I (Orsi, Bakker, et al., 2011). However, the concentration of lineage I was not far from lineage II, and more data and research are needed to understand the ability of lineage I to survive well. In contrast, lineage III has rarely been isolated from food-associated environments (Gianfranceschi et al., 2003), which is why it is not detected in human diseases (Den Bakker et al., 2021). It was underrepresented in the current study's high and lower inocula at the end of storage in the current study, which suggested that it survives poorly on fruit surfaces (Den Bakker et al., 2021).

One study was performed where 80 strains of *L. monocytogenes* were isolated from urban and natural environments in the USA were from lineage II, while urban isolates were evenly split between lineages I and II (Sauders et al., 2006). The same study also examined an additional 921 isolates from farm, food and food processing facilities and clinical samples in combination with the previous 80 isolates to determine lineage differences in samples. Isolates from human clinical samples were usually lineage I, while those from the natural environment, farms, and food were more commonly from lineage II (Sauders et al., 2006; Townsend et al., 2021). That study showed a higher prevalence of lineage I and II than lineage III in the natural and food environment.

One part of trial B was quantitively assessed using the conventional method (MPN-based method). However, when addressing food with a shelf life, the rapid delivery of results is essential (Bernardo et al., 2021). There has been a push for alternatives to conventional culture-based methods; hence the second part of trial B investigated a faster methodology (qPCR).

4.3.2b qPCR method

For each temperature, efficacy for each lineage was evaluated individually through real-time PCR, looking at high (Figure 4.4) and lower inoculum separately (Figure 4.5). As observed with the MPN method, lineage III declined quicker for qPCR than the other lineages for high inoculum on the body regardless of temperatures. Lineage II slightly survived better for high inoculum than lineage I throughout the study. At 0.5°C, lineage II on the body survived better than lineage II in the calyx. Overall, for the body, at the end of the challenge study, lineage I and II survived better than lineage III ($P < 0.05$).

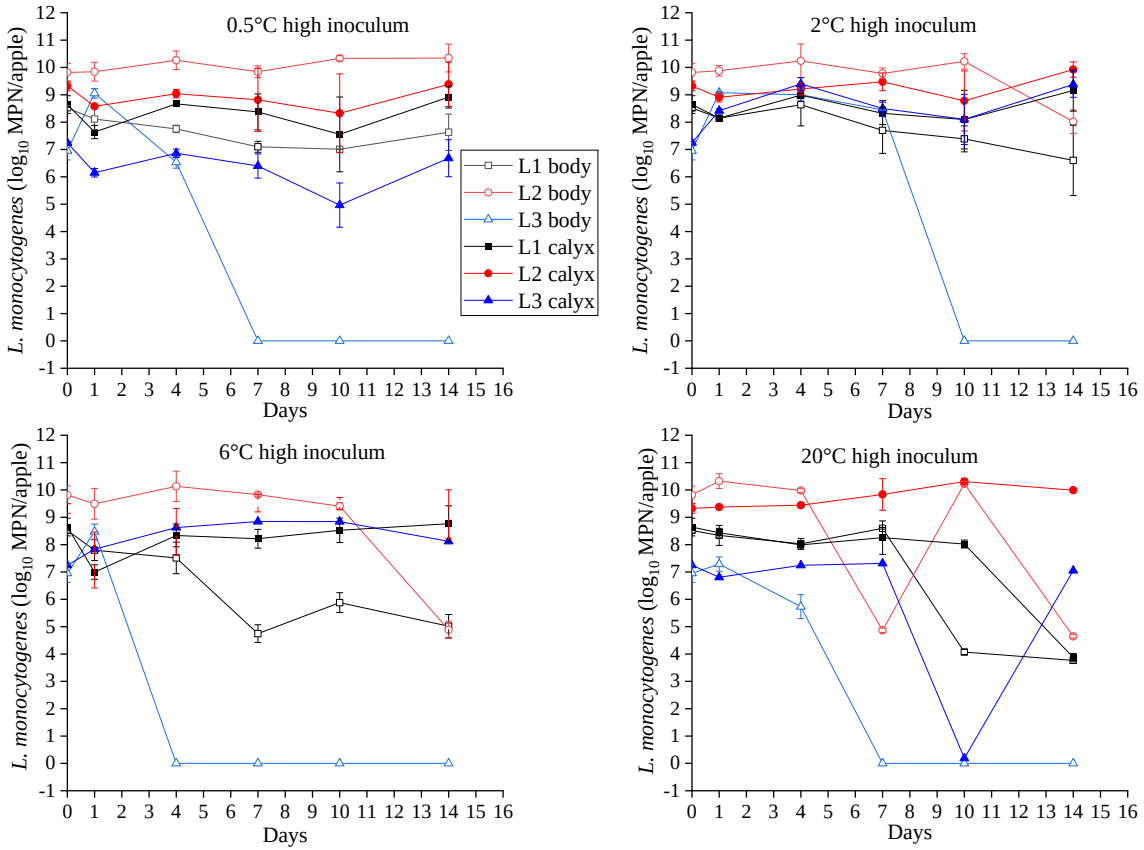

Figure 4.4: Survival of high inocula individual cocktails of *L. monocytogenes* lineages I, II, and III inoculated on the body and in the calyx of the apples under four different static temperature scenarios: 0.5, 2, 6, and 20°C for 14 days, using qPCR.

In Figure 4.4, for the high inoculation in the calyx, growth in the bacterial concentration was observed in some cases. After 14 days' storage, lineage I showed a slight increase of 0.27, 0.51, and 0.12 $\log_{10}$ MPN/apple at storage temperatures of 0.5, 2 and 6°C, respectively. However, the growth was not statistically significant from each other ($P > 0.05$). There was a log reduction of 4.76 $\log_{10}$ MPN/apple at a storage temperature of 20°C. For lineage II, there was also a slight increase of 0.05, 0.60 and 0.68 $\log_{10}$ MPN/apple at storage temperatures of 0.5, 2, and 20°C ($P < 0.05$), respectively. At 6°C, there was a slight drop in the bacteria concentration at 0.21 $\log_{10}$ MPN/apple. For lineage III, there was an increase of 1.43, 2.13 and 0.87 $\log_{10}$ MPN/apple at storage temperatures of 0.5, 2, 6°C ($P > 0.05$), respectively, and a slight drop of 0.18 $\log_{10}$ MPN/apple at 20°C. Across the duration of challenge study, lineages in the calyx were statistically different from the body, with lineage I and II faring better than lineage III ($P < 0.05$). Lineage III at high inoculum in the calyx was not detected on day 10 but reappeared on day 14. The exact reasoning behind this is still unknown; probably a sampling variability.

For high inoculation on the body, after 14 days' storage, for lineage I, a drop of 0.89, 1.91, 3.50, 4.75 $\log_{10}$ MPN/apple was observed at 0.5, 2, 6, and 20°C, respectively. At the end of the study, lineage I was significantly different from the others, except at 2 and 6°C ($P > 0.05$). For lineage II, after 14 days' storage, there was an increase of 0.52 $\log_{10}$ MPN/apple for 0.5°C ($P < 0.05$), and a drop in the bacterial concentration at 1.80, 4.93 and 5.17 $\log_{10}$ MPN/apple for 2, 6, and 20°C ($P < 0.05$), respectively. For lineage III, all the bacteria after 4-10 days (depending on the temperature) dropped below the detection limit of 1.37_{10} log MPN/apple

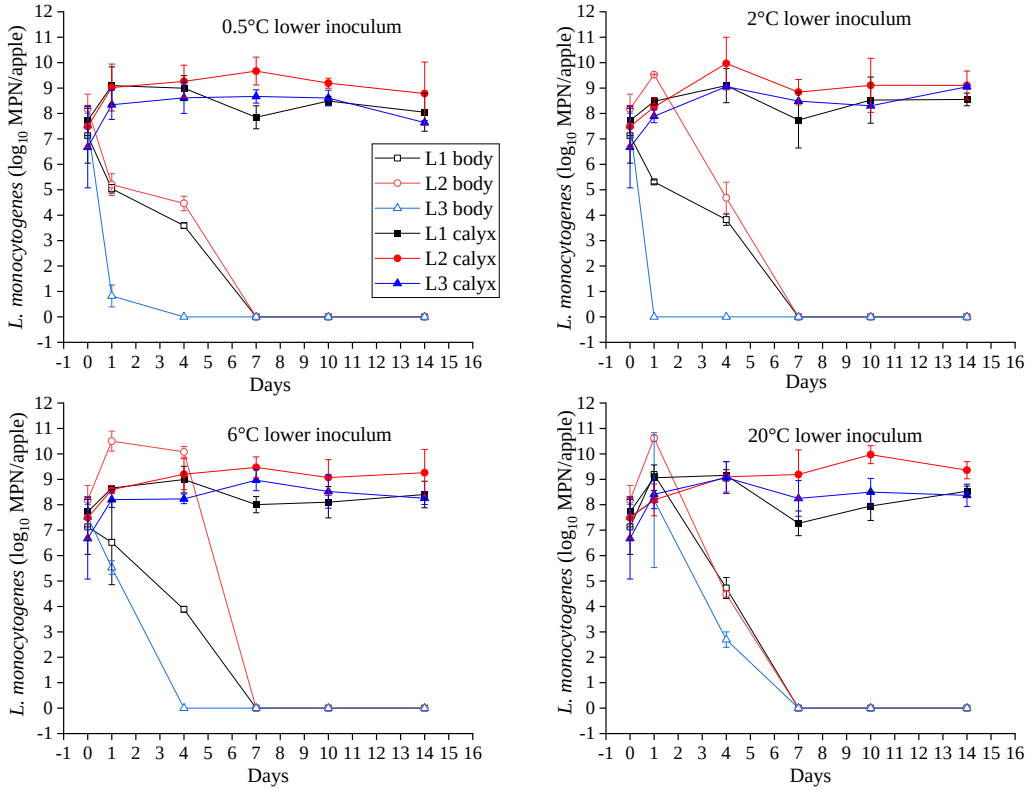

Figure 4.5: Survival of lower inocula of individual cocktails of *L. monocytogenes* lineages I, II, and III inoculated on the body and in the calyx of the apples under four different static temperature scenarios: 0.5, 2, 6, and 20°C for 14 days, using qPCR.

In Figure 4.5, for the lower inocula in the calyx, the bacterial concentration was observed for lineage I, after 14 days' with bacterial growth of 0.92, 1.42, 1.27, 1.40 $\log_{10}$ MPN/apple at 0.5, 2, 6, 20°C, respectively. However, the growth was not significantly different ($P > 0.05$). For lineage II, bacterial concentration increased at 1.30, 1.61, 1.77, 1.87 $\log_{10}$ MPN/apple at 0.5, 2, 6, and 20°C ($P > 0.05$), respectively. For lineage III, an increase in the bacterial concentration at 1.27, 0.48, and 0.59 $\log_{10}$ MPN/apple was observed at 2, 6, 20°C ($P > 0.05$), respectively, and a drop of 0.13 $\log_{10}$ MPN/apple was observed at 0.5°C ($P > 0.05$).

The lower inocula on the body showed little growth on day 1 at higher temperatures (6 and 20°C). All the lineages were below the detection limit after the conclusion of day 7 on the body, which happened in the $\log_{10}$ MPN/apple values in Trial A (Fig. 4.1) as well. The survival of the *L. monocytogenes* in the calyx compared to the body is explained previously in trials A and B. Overall the summary for trials A and B suggested the following learnings.

4.4 Summary results from trials A and B

At the current study's lower temperatures (0.5, 2, and 6°C), *L. monocytogenes* survival for the high and lower inocula for lineage I, II and III on the body or in the calyx were statistically significant, the rate of decline of lineage III was higher than other lineages. This might be due to the desiccation tolerance of lineage I and II compared with lineage III. Desiccation tolerance is the ability of the bacteria to survive on the surface for an extended period with limited availability of nutrients and water (Hingston et al., 2017). In one study, clonal complex CC224 (1/2b) (lineage II) had the highest amount of desiccation survival (Hingston et al., 2017). More importantly, in Denmark, the consumption of deli meat contaminated with clonal complex CC224 resulted in 17 deaths (Kvistholm Jensen et al., 2016). There is a possibility that long-term desiccation survival might be responsible for the occurrence of this outbreak (Hingston et al., 2017). However, there was no evidence that all CC224 strains were

desiccation tolerant. An in-depth study on the role of CC224 in the apple supply chain should be investigated in the future.

Interestingly, the significant difference between trial A and trial B (MPN method) is that in trial A (all the lineages in a single cocktail), the lower inoculum, the *L. monocytogenes* on the body, died in seven days. In contrast, in trial B where the three lineages were inoculated separately, lineage I and II survived 14 days. This might be because the initial inocula applied in trial B was higher (5.8 for lineage I and 5.8 $\log_{10}$ MPN/apple for lineage II) than trial A (4.80 $\log_{10}$ MPN/apple), showing inter-strain competition could lower the bacterial load. For trial B, both qPCR and MPN data showed that lineages I and II survived, and both types of inocula were statistically different ($P < 0.05$). Previous cold storage studies have found serotype 1/2a (lineage II) to be colder tolerant than serotype 4b (lineage I) (Buncic et al., 2001; Hingston et al., 2017; Lianou et al., 2006). Studies have suggested that lineage II strains may be able to survive better under food-related stress due to their superior ability to develop mutations and extrachromosomal DNA compared to lineage I strain, which have more conserved genomes (Dunn et al., 2009; Hingston et al., 2017; Orsi, Bakker, et al., 2011; Orsi et al., 2008). Compared to lineage I, stress response genes are mainly present in membrane transport and cell wall structure in lineage II (Doumith et al., 2004). These cell wall structures play a crucial role in the ability of bacteria to adapt under stress. Different *L. monocytogenes* lineages and serotypes can behave differently under different types of stresses (Annous et al., 1997; Hingston et al., 2017).

While comparing Fig. 4.2 and 4.3 (MPN method) with 4.4 and 4.5 (qPCR method), respectively, there is a difference in the survival of *L. monocytogenes* lineages. The qPCR detected more survival of the *L. monocytogenes* lineages than the culture-based MPN. This reflects the nature of the methods. The conventional MPN method detects only living cells, whereas qPCR detects nucleic acids rather than living cells. Living and dead cells both have

nucleic acid. The nucleic acids originating from dead cells may interfere with the final results (Scheu et al., 1998; Wolffs et al., 2005), leading to an overestimation of the bacterial concentration (Bernardo et al., 2021). Propidium monoazide (PMA) prior to DNA extraction could be used to identify viable and dead cells in the apple supply chain. This method is based on the presence of an azide group that cross-linked the dye to the DNA of dead cells. The induced DNA modification will inhibit the increase in subsequent PCR reactions, while the DNA of viable cells protected by intact membranes could be detected by qPCR (Bernardo et al., 2021). However, as the dead cells could also be present in the intact membrane, one must be cautious when using PMA qPCR for food safety (Auvolat and Besse, 2016; Bernardo et al., 2021).

From the above discussions, more research is needed to explore whether nonculture-based assays allow discrimination between viable and nonviable organisms (Norton, 2002). Messenger RNA (mRNA) is a promising target for detection as it is produced only by viable organisms. Additionally, gene expression results in the production of multiple mRNA copies, resulting in an increased number of target sequences and assay sensitivity (Norton, 2002). Reports are available on developing nucleic acid amplification-based assays for *L. monocytogenes* targeting RNA (Blais et al., 1997; Herman, 1997; Klein and Juneja, 1997; Norton and Batt, 1999).

4.5 Conclusion

This study showed that *L. monocytogenes* lineages behaved differently in different temperatures under static temperature conditions. The *L. monocytogenes* survival is moderately temperature-dependent, with better survival at lower storage temperatures. If *L. monocytogenes* is present in the calyx, or probably in any hidden structure of the apple, its survival will be enhanced compared to open structures like skin/body. Based on this research, those consumers who eat the core of an apple might need to be extra vigilant if they are at

risk from listeriosis. Another important finding is that lineage III did not survive in MPN or qPCR quantification methodologies compared with lineage I and II. Also, as qPCR could end up getting the results with both live and dead bacterial nucleic acids, a rapid detection methodology like mRNA detection should be looked in the future.

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Preface to Chapter 5

L. monocytogenes is an opportunistic bacterium that can colonise various environments and food surfaces. As apples have various micro and macrostructures, these structures can act as harbourage sites for *L. monocytogenes* to survive. This bacterium can form spatially organised communities, and its ability to form structured communities (as biofilms) could contribute to its survival.

As *L. monocytogenes* could behave differently at different temperatures, its ability to form biofilms could also be different for temperatures, including lineage differences. The effect of apples at different temperatures on individual lineages and their attachment on the apple surface and calyx is unknown, hence the research investigated in this chapter is important.

Chapter 5: The effect of commercial storage temperature on the survival and microscopic attachment of three lineages of *Listeria monocytogenes* on two apple cultivars

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Abstract

Temperature plays an essential role in the survival of *Listeria monocytogenes (L. monocytogenes)*. There are four lineages of the *L. monocytogenes* family, and no quantified literature is available to assess its role in apples stored under different temperatures.

The objective of this study was to investigate the effect of commercial storage temperatures (0.5, 2, 6, and 10°C) on the attachment and survival of three lineages of *L. monocytogenes* on two apple cultivars (‘Royal Gala’ and ‘Scired’) over two weeks of storage. The apples were inoculated on the body and in the calyx of the fruit with 10^8 cells of a cocktail containing two strains of each of three lineages (I, II and III). The apples were sampled and enumerated for *L. monocytogenes* after 0, 1, 4, 7 and 14 days of storage. Scanning electron microscopy (SEM) was also used to understand the attachment mechanism on samples (on day 0, and 14 days). Results showed that there were significant differences in survival of the three *L. monocytogenes* lineages' across all the temperatures. *L. monocytogenes* lineage I and II

survived better than lineage III in both inoculation regions of both apple cultivars. Temperature and lineage notwithstanding, *L. monocytogenes* survived better in the calyx than on the body. No *L. monocytogenes* growth was observed on apples under any test conditions. The effect of cultivar on the survival of *L. monocytogenes* was statistically insignificant for all the lineages, while the region of inoculation had a statistically significant effect. SEM results suggested that to adapt to various stresses, especially temperature, *L. monocytogenes* made biofilms, more in the calyx than on the body. SEM images showed that the capacity to make biofilms was higher for lineage II than for lineage I and III. However, more studies are needed to verify these results, to mitigate the risks posed by *L. monocytogenes*, especially from lineage I and II, which survived best in the harsh conditions used in this study.

Keywords: SEM – Scanning electron microscope, temperatures, attachment, apple body and calyx, biofilm

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5.1 Introduction:

The apple industry is at the heart of the horticulture world economy and one of the world's oldest permanent crops (O'Rourke, 2021). Apple production in the southern hemisphere, especially in New Zealand is export market-centric (O'Rourke, 2021), and is considered the key part of New Zealand's economy.

Apples were once considered to be the safest fresh produce to eat. However, this perception was challenged by an incident due to *L. monocytogenes* in 2014/2015, in which 35 people across 12 U.S. states contracted listeriosis, 7 of whom died (Angelo et al., 2017; CDC, 2015, 2016, 2021; Nangul et al., 2021). This outbreak shows the urgency to improve current food safety systems in the apple packing industry (Guan et al., 2021), especially in understanding the behaviour of *L. monocytogenes* in the apple supply chain.

L. monocytogenes is a gram-positive, rod-shaped, motile, pathogenic and facultative anaerobic bacterium (Liu, 2008), which is persistent in the cold environment (Guan et al., 2021; Walker et al., 1990). *L. monocytogenes* is also responsible for causing listeriosis, with a mortality rate of 20% to 30% (CDC, 2021; USFDA, 2020). *L. monocytogenes* can rapidly adapt to changing environmental conditions, which enables it to survive harsh environments during food processing (Henderson et al., 2019). Contamination can occur during any stage of the supply chain, from preharvest to postharvest. During postharvest processing in the packhouse, postharvest washing is a wet-cleaning operation that removes physical contaminants (e.g., soil, fine particulates, orchard litter). It may have some benefit in removing microbial contaminants (Murray et al., 2017), but even when sanitisers are used, it is a risk reduction step, not a critical control point. Washing could potentially lead to cross-contamination, which helps *L. monocytogenes* adapt to produce associated environments, e.g., cold storage (Du et al., 2002).

In the past two decades, listeriosis due to *L. monocytogenes* contamination outbreak/recalls associated with fresh produce has increased (Botticella et al., 2013), e.g. apples (FDA, Food and Drug Administration 2020), melons (CDC, 2012) and recently rock melons in Australia, which resulted in seven deaths and one miscarriage (DPI, 2018). One of the primary reasons for these outbreaks is that the fresh produce is eaten raw.

In a typical apple packing process, the apples are submerged in the bins in a water dump tank. The apples float of the bins in the dump tank as the bin is removed from the dump tank (Pietrysiak and Ganjyal, 2018). Next, the apples are carried by water flumes, which help remove leaves and organic debris. Water in the dump tank and flumes is treated with sanitation chemicals, such as chlorine, HarvestCide® (Postharvest Solutions, Whakatu, NZ) or peracetic acid (PAA). Constant introduction of new organic matter, water recirculation, and size of the dump tank and flume reduce the effectiveness of the water sanitising

treatments, leading to an increased risk of apple contamination with pathogenic bacteria (Artes et al., 2009; Parish et al., 2003). Once contaminated, it is challenging to reduce or eliminate a pathogenic bacteria like *L. monocytogenes* from apples (Zhu and Suslow, 2018), as the morphology of apple fruit has a lot of hidden structures like stem and calyx where this contaminated water with the bacteria can reside (Pietrysiak and Ganjyal, 2018).

The apple morphology susceptible to bacterial contamination can be described at three levels: (1) overall shape; (2) presence of macrostructures (i.e. stem bowl, stem, and calyx); and (3) surface microstructures (i.e. epicuticular waxes, trichomes, microcracks, lenticels, and stomata) (Pietrysiak and Ganjyal, 2018). The presence of macrostructures such as stem end, and calyx may provide more harbour-sites for bacteria that are difficult to clean in the packhouse if the apples are contaminated (Buchanan et al., 1999; Kenney et al., 2001). Apple morphology mentioned above could help opportunistic bacteria like *L. monocytogenes* attached to the apple at various levels of the supply chain, e.g. when the apples are picked by hand in the orchards, transported in the bins to the pack-house, they get either cold-stored or washed, sized, sorted, and packed for the retail market (Pietrysiak et al., 2019). The other opportunity for *L. monocytogenes* to get attached to the apple could be when freshly picked apples are stored in cold storage before being washed and packaged (Guan et al., 2021).

For *L. monocytogenes* to get attached to the apple, temperature plays a critical role, although attachment of *L. monocytogenes* is affected by several other factors such as bacteria features, produce surface properties, and exposure time (Reina et al., 2002). Previous literature suggested the molecular mechanism of *L. monocytogenes* attachment (Gorski et al., 2003; Reina et al., 2002) and internalisation (Chen et al., 2016; Macarisin et al., 2017) on the surface of other fresh produce. However, the microscopic attachment of *L. monocytogenes* to the surface of apples and in the calyx has yet to be widely studied (Pietrysiak and Ganjyal, 2018), including attachment studies for individual *L. monocytogenes* lineages at different

temperatures. There is a significant knowledge gap in understanding the apple surface and its hidden structure's morphology at the microscopic level. Understanding the locations where *L. monocytogenes* could hide or attach could help develop more effective plans to reduce the microbial load (Pietrysiak and Ganjyal, 2018).

L. monocytogenes consists of four lineages: I, II, III, and IV (Piffaretti et al., 1989; Rasmussen et al., 1995; Roberts et al., 2006; Ward et al., 2008; Wiedmann et al., 1997). Most *L. monocytogenes* isolates belong to lineages I and II, which contain serotypes more commonly associated with human clinical cases, including serotype 1/2a (lineage II) and serotypes 1/2b and 4b (lineage I) (Orsi, Bakker, et al., 2011). Lineage II strains are common in food products, widespread in natural and farm environments, and are less frequently isolated from human clinical cases (Kabuki et al., 2004; Manfreda et al., 2005). Most human listeriosis outbreaks are associated with lineage I isolates (Jeffers et al., 2001). Lineage III and IV strains are rare in the packhouse facility and predominantly isolated from animal sources (Jeffers et al., 2001; Wiedmann et al., 1997). The effect of different temperatures on *L. monocytogenes* cocktails in apple cultivars has been quantified before (Nangul et al., 2021; Sheng et al., 2017), which showed that temperature could affect the growth rate of bacteria on fresh produce surfaces (Danyluk et al., 2012). However, the effect of apples at different temperatures on individual lineages and their attachment to the apple surface and calyx is unknown. Therefore, this study aimed to understand the effect of temperature on the survival of three individual *L. monocytogenes* lineages (lineage IV isolates were not available to include in the challenge study). This will be achieved by understanding the following:

i. Effect of two apple cultivars at different inoculated locations - body and calyx on the individual lineages of *L. monocytogenes,* when stored at different temperatures.

ii. Microscopic attachment of individual *L. monocytogenes* lineages on the apple cultivars at different inoculated regions using a scanning electron microscope (SEM) when the apples were stored at different temperatures.

5.2 Materials and methods

5.2.1 Apples

Commercially graded 100 count size for 18 kg carton export quality un-waxed apples (*Malus* x *domestica*) were sourced from a commercial New Zealand packhouse. Open and closed calyx cultivars were chosen, where 'Scired' and 'Royal Gala' represented the open and closed calyx cultivars, respectively. The fruit had been through conventional packhouse treatments including the use of sanitisers. Apples with no bruising, cuts or scars were selected and used for the study.

5.2.2 Listeria monocytogenes strains

To account for variation in growth and survival rates amongst bacterial strains, six genetically different *L. monocytogenes* isolates of different lineages isolated from horticultural sources (PFR46G06, PFR46E10, PFR40I07, PFR41I04, PFR41F08, PFR41H05) (Plant & Food Research, Auckland, New Zealand) were chosen to make a bacterial cocktail for each lineage based on the strains below (Table 5.1).

Table 5.1: Lineage of every *L. monocytogenes* strain used to make the bacterial cocktail. The explanation of every lineage and its sequence type is already described in (Nangul et al., 2021).

***L. monocytogenes* strains**	**Lineage**
PFR46G06	I
PFR46E10	I
PFR40I07	II
PFR41I04	II
PFR41F08	III
PFR41H05	III

5.2.3 Inoculum Preparation

L. monocytogenes strains were assessed for purity by streaking them on tryptic soy agar with 0.6% added yeast extract (TSAYE) (Becton, Dickinson & Company, USA) and then onto *Listeria* CHROMagar™ plates (CHROMagar™, Paris, France). The resulting colony morphologies were assessed for appearance and uniformity. Pure bacterial cultures were then grown in tryptic soy broth with 0.6% added yeast extract (TSBYE) (Becton, Dickinson & Company, USA) for 48 h at 37°C to achieve a stationary growth phase. The strains were then centrifuged at 4000 rpm (3220g) (model 1736R, Gyrozen, Seoul, South Korea) for 10 min, at 4°C. The resulting pellets were washed twice with 0.1% peptone (10 mL). An equal amount of each *L. monocytogenes* strain was combined to make a two-strain cocktail for each lineage (lineage I, II, and III), respectively, with a final concentration of 10^8 CFU/mL in 0.1% peptone.

5.2.4 Inoculation of apples

Whole unwaxed apples were removed from cold storage (0.5°C) and left at room temperature (20°C) overnight before inoculation to equilibrate the apple temperature. For each cultivar and lineage, apples were spot-inoculated with *L. monocytogenes*. The inoculation was divided into two groups: (i) calyx inoculation - 50 µL of *L. monocytogenes* cocktail was pipetted into the calyx of each apple and (ii) body inoculation - two droplets of 25 µL were pipetted onto the equatorial region. The apples were air-dried for three hours in a biosafety class II cabinet at room temperature until visibly dry.

5.2.5 Apple storage and sampling

The apples were inoculated with a two-strain cocktail of *L. monocytogenes* individual lineages and then stored in a temperature-controlled room at 0.5, 2, 6 or 20°C. Apples were sampled after air drying (day 0) and after 1, 4, 7, 10, and 14 days of storage. At each time

point, samples comprised nine fruits (three sets of three apples) of each combination of lineage, inoculation site, and temperature.

5.2.6 Bacterial enumeration from apples

For body-inoculated samples, apples were placed into a sterile bag with 400 mL of Buffered *Listeria* Enrichment Broth (BLEB) (Acumedia, Lansing, Michigan, USA) and hand massaged for two min. Triplicate aliquots of 2 mL BLEB wash solution from each bag were transferred into 15 mL falcon tubes. Triplicate aliquots of 200 µL were dispensed in a 96-well microtiter plate, followed by ten-fold serial dilution in BLEB. All the aliquots and the original bag were then enriched by incubating for 48 h at 30°C. After 48 h, 2 µL solution from each turbid bag, tube, and micro-titre well was plated onto pre-gridded selective CHROMagar™ *Listeria* plates. After the plates were incubated for 48 h at 37°C, blue colonies were presumptively identified as *L. monocytogenes*.

For calyx-inoculated samples, the core of each apple was taken out with a sterile cork-borer (16 mm diameter), weighed, and homogenised in a laboratory stomacher (Smasher, AES Chemunex, AES laboratory, France) for two minutes in BLEB (1:10). After this, the homogenate followed a similar protocol used for enumeration on the body inoculation mentioned above. Three sets of three apples were quantitatively assessed for *L. monocytogenes* by sampling the body and the calyx using a most probable number (MPN) method to detect stressed organisms (Osborne and Bremer, 2002). The MPN values were calculated using the Bacteriological Analytical Manual (BAM) spreadsheet (Blodgett, 2010). All results were expressed as $\log_{10}$ MPN per apple with a detection limit of 1.37 $\log_{10}$ MPN/ apple from the tubes and wells and presence/absence in three apples from the bags.

5.2.7 Scanning electron microscopy (SEM)

Apple pieces (about 2 x 2 x 2 mm) were gently cut from the inoculated region of the body and calyx with a sterile razor blade. The cut pieces were transferred into a vial with 0.1M

phosphate buffer solution (PBS) and primary fixative (2.5% glutaraldehyde). The solution was incubated for at least 24 h. The glutaraldehyde was removed, and the specimens were rinsed twice in 0.1 M PBS (pH 7.2) and once in sterile water. A series of incubation steps followed, where the samples were soaked in 30% ethanol for 10 min, twice, followed by 50% ethanol (2 x 10 min), 70% ethanol (2 x 10 mins), 95% ethanol (2 x 10 min), 100% ethanol (2 x 10 mins). Then, all the samples reached for critical-point dry (BalTec CPD030, Balzers, Lichtenstein). After this, samples were mounted onto SEM stubs, coated with gold when mounted on a sputter coater (Leica EM ACE200, Leica Microscopy Systems ltd., Switzerland), and viewed under the SEM (Quanta 250 scanning electron microscope, FEI Company, Hillsboro, Oregon, USA). Samples were examined at a magnification range of 6000 X to identify any unique surface characteristics and confirm the presence of *L. monocytogenes*.

5.2.8 Statistical analysis

Microbial MPN data was analysed using GenStat (version 18th; VSN International Ltd), where all the data was analysed using analysis of variance (ANOVA) followed by a post-hoc Tukey's test. Results were presented as means with standard deviations in the graphs. A value of $P < 0.05$ was considered statistically significant. All the figures were prepared using Origin software 2021b (OriginLab Corporation, Northampton, USA).

5.3 Results and discussions

5.3.1 Microbial data

Table 5.2 showed the concentrations of different lineages of *L. monocytogenes* on apples at the start and end of 14 days of storage at 0.5, 2, 6, and 20°C.

At 0.5°C, every lineage of *L. monocytogenes* survived significantly better in the calyx than the body ($P < 0.05$; Table 5.2). Lineages I and II survived with significantly higher counts

than lineage III ($P < 0.05$). No cultivar effect on the body and calyx inoculations within the lineages were found at 0.5°C.

At 2°C, regardless of cultivar, lineages I and II survived better than III in the calyx were statistically different ($P < 0.05$; Table 5.2), with lineage III concentration surviving the least.

Table 5.2: Concentrations of different lineages of *Listeria monocytogenes* on apples at the start and end of 14 days of storage at 0.5, 2, 6, and 20°C. All the data with the same letters in the superscript of each temperature are not significantly different (P > 0.05), while those with different letters are different (P < 0.05) from each other. Column heading with 'Lin.' denotes lineages of *L. monocytogenes*. Column heading with 'Inoc. region' should be read as 'Inoculation region'. The apple cultivar RG denotes 'Royal Gala' in the variety column. Column heading 'Red.' should read as 'Log reduction'.

Lin.	Inoc. region	Cultivar	(log_{10} MPN/apple)											
			0.5°C			2°C			6°C			20°C		
			Initial	Final	Red.	Initial	Final	Red.	Initial	Final	Red.	Initial	Final	Red.
I	Calyx	RG	7.30^{h}	5.43^{e}	1.87	7.30^{kl}	5.14^{fg}	2.16	7.30^{h}	4.64^{d}	2.66	7.30^{ij}	4.32^{e}	2.98
		'Scired'	7.30^{h}	5.30^{e}	2.01	7.38^{l}	5.03^{ef}	2.35	7.39^{h}	4.65^{d}	2.74	7.40^{j}	4.33^{e}	3.07
II		RG	7.40^{h}	5.62^{e}	1.78	7.40^{l}	5.52^{gh}	1.88	7.39^{h}	5.31^{e}	2.08	7.50^{j}	5.32^{f}	2.18
		'Scired'	7.38^{h}	5.65^{e}	1.73	7.38^{l}	5.63^{h}	1.75	7.37^{h}	5.31^{e}	2.06	7.37^{j}	5.25^{f}	2.12
III		RG	7.50^{h}	3.67^{b}	3.84	7.50^{l}	3.43^{b}	4.07	7.50^{h}	3.11^{b}	4.39	7.50^{j}	2.77^{b}	4.73
		'Scired'	7.38^{h}	3.65^{b}	3.73	7.38^{l}	3.63^{c}	3.75	7.38^{h}	3.30^{b}	4.08	7.38^{j}	3.16^{bc}	4.22
I	Body	RG	6.89^{g}	4.36^{c}	2.52	6.89^{jk}	4.27^{cd}	2.62	6.89^{g}	3.79^{c}	3.10	6.89^{hi}	3.45^{cd}	3.44
		'Scired'	6.27^{f}	4.45^{cd}	1.82	6.27^{i}	4.45^{cd}	1.82	6.27^{f}	3.79^{c}	2.48	6.27^{g}	3.45^{cd}	2.82
II		RG	6.91^{g}	4.45^{cd}	2.46	6.91^{jk}	4.19^{c}	2.72	6.89^{g}	3.79^{c}	3.10	6.90^{hi}	3.78^{d}	4.41
		'Scired'	6.89^{g}	4.79^{d}	2.10	6.89^{jk}	4.67^{de}	2.22	6.87^{g}	3.79^{c}	3.08	6.89^{hi}	3.67^{d}	3.22
III		RG	6.59^{fg}	2.19^{a}	4.40	6.59^{ij}	2.19^{a}	4.40	6.59^{fg}	2.19^{a}	4.40	6.60^{gh}	2.19^{a}	4.41
		'Scired'	6.27^{f}	2.27^{a}	4	6.27^{i}	2.19^{a}	4.08	6.27^{f}	2.19^{a}	4.08	6.27^{g}	2.19^{a}	4.08

At 6°C, no cultivar difference was observed. In the calyx, every lineage differed significantly from the other ($P < 0.05$). For body, lineage I and II fared equally ($P > 0.05$) and were significantly different from lineage III ($P < 0.05$), with lineage III having the most significant drop in the concentration.

For 20°C after 14 days, like other temperatures, there was no cultivar effect on the body and calyx for either of the lineages of *L. monocytogenes* (Table 5.2). For the calyx inoculation, all three lineages were significantly different from each other ($P < 0.05$), a similar result was found for body inoculations as well.

Figure 5.1 below shows the survival of *L. monocytogenes* lineage I, II and III on the body and calyx of apples during 14-day storage at 0.5, 2, 6, and 20°C. Overall, at 0.5°C (Fig. 5.1A), after the end of storage, the MPN data for lineage I and II were similar ($P > 0.05$), and both the lineages were statistically different from lineage III ($P < 0.05$).

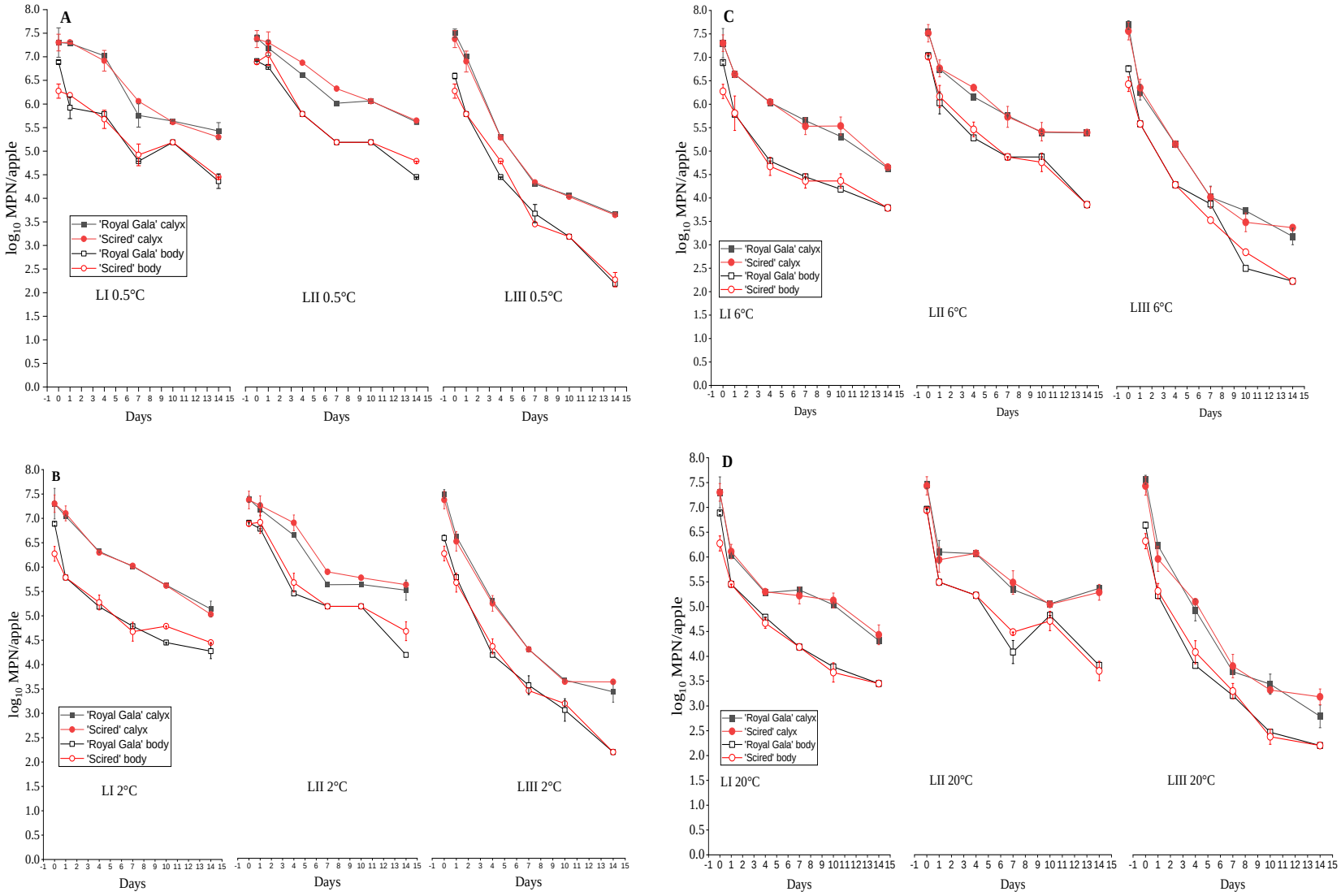

Figure 5.1: Survival of inoculated lineages I, II and III of *Listeria monocytogenes* on the body and calyx of two apple varieties during 14-day storage for temperature of (A) 0.5°C, (B) 2°C, (C) 6°C, and (D) 20°C

During 14-day storage for a temperature of 2°C, overall at 2°C, lineage I and II survived significantly better than lineage III ($P < 0.05$; Fig. 5.1B). Also, like 0.5°C, every lineage of *L. monocytogenes* survived significantly better in the calyx than the body ($P < 0.05$). Different cultivars did not affect the survival of every lineage of *L. monocytogenes* at each inoculation region ($P = 0.623$).

Overall at 6°C, after the study, all three lineages in the calyx were significantly different ($P < 0.05$; Fig. 5.1C). Lineages I and II on the body survived better than lineage III. Also, similar to 0.5 and 2°C, every lineage of *L. monocytogenes* survived significantly better in the calyx than the body ($P < 0.05$). The survival of *L. monocytogenes* on both varieties was statistically insignificant ($P = 0.442$), and the effect of *L. monocytogenes* on the two varieties at different inoculation regions was not significantly different from each other ($P = 0.061$).

Overall, at 20°C, after the study's conclusion, all three lineages in the calyx or the body were significantly different from each other ($P < 0.05$; Fig. 5.1D). Also, every lineage of *L. monocytogenes* survived significantly better in the calyx than the body ($P < 0.05$), like 0.5, 2 and 6°C. The survival of *L. monocytogenes* in both varieties was insignificant ($P = 0.442$), and the effect of the two varieties on *L. monocytogenes* at different inoculation region was not significantly different from each other ($P = 0.061$).

Also, the MPN data for all three lineages at different inoculation regions were significantly different from each other as well ($P < 0.05$). The effect of *L. monocytogenes* on the two varieties at different inoculation region was not significantly different from each other ($P = 0.483$).

Overall, Table 5.2 and figures (Fig.) 5.1A, 5.1B, 5.1C, 5.1D suggested that the cultivar showed no statistical difference ($P > 0.05$) for all the lineages of *L. monocytogenes*, either in the calyx or on the body. For all the temperatures in this study and all lineages, *L.*

monocytogenes survived better in the calyx than on the body ($P < 0.05$). The unavailability of nutrients on the body than in the calyx, and the opportunity for the bacteria to hide in the crevices of the calyx may be reasons for the higher log reduction of bacteria on the body than in the calyx (Nangul et al., 2021; Pietrysiak and Ganjyal, 2018).

From all the MPN data, *L. monocytogenes* did not grow on the temperatures used in this study and were significantly different from each other ($P < 0.05$). *L. monocytogenes* under cold conditions (especially for the temperatures used in this study) adapts itself according to the environment. In cold environments, due to the psychrotrophic nature of *L. monocytogenes*, it uses unique adaptation mechanisms, including stabilising ribosomes that lose stability at low temperatures (Garmyn et al., 2012; Saldivar et al., 2018; Tasara and Stephan, 2006).

The regulation of cold shock protein is a key adaptation system. Cold shock protein (Csp) family are some of the significant regulators of that adaptation system (Muchaamba et al., 2021). Csps not only help *L. monocytogenes* to survive but also helps in virulence, i.e. cell invasion (Muchaamba et al., 2021). Csps are the proteins that help bind the RNA and DNA, which helps the bacteria regulate gene expression during virulence and stress responses (Loepfe et al., 2010; Schärer et al., 2013). Csps are highly conserved regions between different genetic backgrounds in *L. monocytogenes* (Muchaamba et al., 2021). The sequence identity of Csps between the amino acid sequence in *L. monocytogenes* is 67% to 73% (Schmid et al., 2009). Under cold stress conditions, there is a decrease in the cell membrane fluidity and enzyme activity and transcription and translation decrease (Zhang et al., 2018). Although different types of cold shock proteins are available e.g. CspA, CspB, CspD, but CspA is more critical under cold stress conditions (Muchaamba et al., 2021). Under cold stress conditions (4°C), CspA gene expression was more upregulated than CspB and CspD

(Schmid et al., 2009). As the bacteria did not grow even at 20°C, it is hypothesised that a similar mechanism may have happened at that temperature.

Another adaptation mechanism is altering the branching in the methyl end of the fatty acid from iso to anteiso by shortening the length of fatty acid chains (Annous et al., 1997). This adaptation mechanism helps retain the membrane fluidity, an essential survival aspect (Saldivar et al., 2018).

In this experiment, lineages I and II across all the temperatures were significantly different ($P < 0.05$) from Lineage III, for both the inoculation regions. Lineages I and II behaved similarly at different temperatures in this study, which is quite inconsistent with the literature. *L. monocytogenes* can grow at various temperatures, but every strain/lineage behaves differently in its ability to adapt to cold stress (Hingston et al., 2017). Literature suggested lineage II strains survive better in food-related cold stresses than lineage I. There are a few reasons behind that, for example, lineage II has more extrachromosomal DNA with conserved genomes than lineage I (Orsi et al., 2008). Some stress response genes are mainly involved in membrane transport and cell wall structure in lineage II and absent in lineage I (Borucki and Call, 2003; Doumith et al., 2004), including the lmo1078 gene containing UDP-glucose pyrophosphorylase (Chassaing and Auvray, 2007). Lineage II food isolates have genes *cadAC*, *ebrB,* and *qac,* which help survive in the food and food processing environment by slowing cell exposure to harmful chemicals. The three previously mentioned genes (*cadAC, ebrB, qac*) were not found in lineage I and III (Pirone-Davies et al., 2018). *sigB* gene was overexpressed in lineage II isolates, potentially reassuring that this lineage could inhabit different environments and unfavourable conditions like food processing conditions (Orsi, Bakker, et al., 2011). Plasmid harbourage is another phenomenon that overexpresses in lineage II. Literature suggests that plasmid harborage, following replication in a bacteria, increases the cells' metabolic demand, resulting in a decreased growth rate than the plasmid-

free strains (Diaz Ricci and Hernández, 2000). However, these functions depend on the type of genes any plasmid carries, which could potentially result in improved acid tolerance and cold sensitivity. Research on 166 *L. monocytogenes* isolated from Canada and Switzerland showed that lineage II isolates showed a higher level of plasmid harborage than lineage I (Hingston et al., 2017). The current study showed an equal representation of lineage I and II, indicating a perception of overrepresentation of lineage I strain in human listeriosis cases than its prevalence in food appears inconsistent and should be looked at in the future.

At the end of the study for each temperature, the concentration of every lineage of *L. monocytogenes* did not increase. However, it did not die-off completely. Literature suggests that desiccation stress might be responsible for stress tolerance. The bacteria can survive on the surface for long periods with very limited nutrient and water availability (Hingston et al., 2017). Although very little research has been done on the desiccation stress of *L. monocytogenes*, work done on isolates showed that, in general, serotype 1/2c (lineage II) and 1/2b (lineage I) were more desiccation tolerant than the others (Hingston et al., 2017; Kvistholm Jensen et al., 2016). That might be the reason for the better survival of lineage I and II than lineage III.

While discussing lineage III, lineage III strains, on average, are less suited to survive in stressful conditions with food environments (Roberts et al., 2006). A study on the thermal inactivation and growth characteristics of lineage I, II, and III isolates found that lineage III isolates are least resistant to heat and cold stress than lineage I and II (De Jesús and Whiting, 2003). However, another research showed that lineage III has the full potential to grow in normal conditions and can be virulent once consumed (Roberts et al., 2006). The current temperatures (0.5, 2, 6 and 20°C) used in the challenge study clearly show that as lineage III performed the least compared with lineage I and II, these temperatures were not regarded as a normal condition to grow for lineage III.

5.3.2 Attachment of L. monocytogenes through scanning electron microscopy (SEM)

The individual lineage (lineage I, II and III) of *L. monocytogenes* attachment on two apple cultivars ('Royal Gala' and 'Scired') were studied on the body and in the calyx at different temperatures (0.5, 2, 6, 20°C), over two weeks. Figures 5.2 - 5.5 shows the bacterial attachment of individual lineages of *L. monocytogenes,* where Figure 5.2 shows the attachment on the body of the 'Royal Gala' apple, Figure 5.3 is on the body of the 'Scired' apple, Figure 5.4 is the calyx of 'Royal Gala' apple and Figure 5.5 is the calyx of 'Scired' apples.

Due to dense microstructures in the calyx, the bacteria of every lineage were more challenging to locate in the calyx than on the body. For both cultivars, every lineage of *L. monocytogenes* could attach to the body and in the calyx, more so to the calyx. Similar behaviour was observed when *L. innocua* was attached to the epidermal cell exposed in the lenticel wall and trichomes, and the internal surface of microcracks covered with different forms of epicuticular wax (Pietrysiak and Ganjyal, 2018). This observation highlighted the influence of apple microstructures on bacterial attachment. Microstructures such as microcracks, lenticels or trichomes may serve as harbour sites for *Listeria* through physical entrapment (Pietrysiak and Ganjyal, 2018). The SEM pictures from Figures 5.2 -5.5 showed *L. monocytogenes* lineages attached primarily to the calyx than on the smooth body surface with the waxy layer.

Bacteria attach to the surface of the fruit using two steps; the first step includes the primary cells getting connected to the surface by physical forces such as Van der Waals forces, gravitational forces, and hydrophobic surface interactions due to electrostatic charges (Katsikogianni and Missirlis, 2004). The strength of the bacterial attachment is influenced by the hydrophobic forces and charge it carries on the surface (Pietrysiak and Ganjyal, 2018), e.g. *L. monocytogenes* had a higher attachment on the cut edges of the iceberg lettuce than the

intact surface (Takeuchi et al., 2000). The second step includes stronger surface binding through exopolymer substances (EPS) (Combrouse et al., 2013; Hori and Matsumoto, 2010; Pietrysiak and Ganjyal, 2018). Exopolymer substances form the structure and architecture of the biofilm. The primary role of EPS is facilitation of the initial attachment of cells to different substances and protection against environmental stress (Vu et al., 2009). Bacteria also attached heavily to the internal structures of the apples, such as trichomes, and microcracks, where the highest levels of contamination is usually found. A study on *E. coli* 0157:H7 found that the bacteria attached to the internal trichomes within the floral tube can infiltrate through the calyx to the inner core of the fruit (Burnett et al., 2000). Compared to the smoother surfaces, More bacterial attachments were found with trichomes and microcracks in apples (Pietrysiak and Ganjyal, 2018) and leafy greens (Patel and Sharma, 2010).

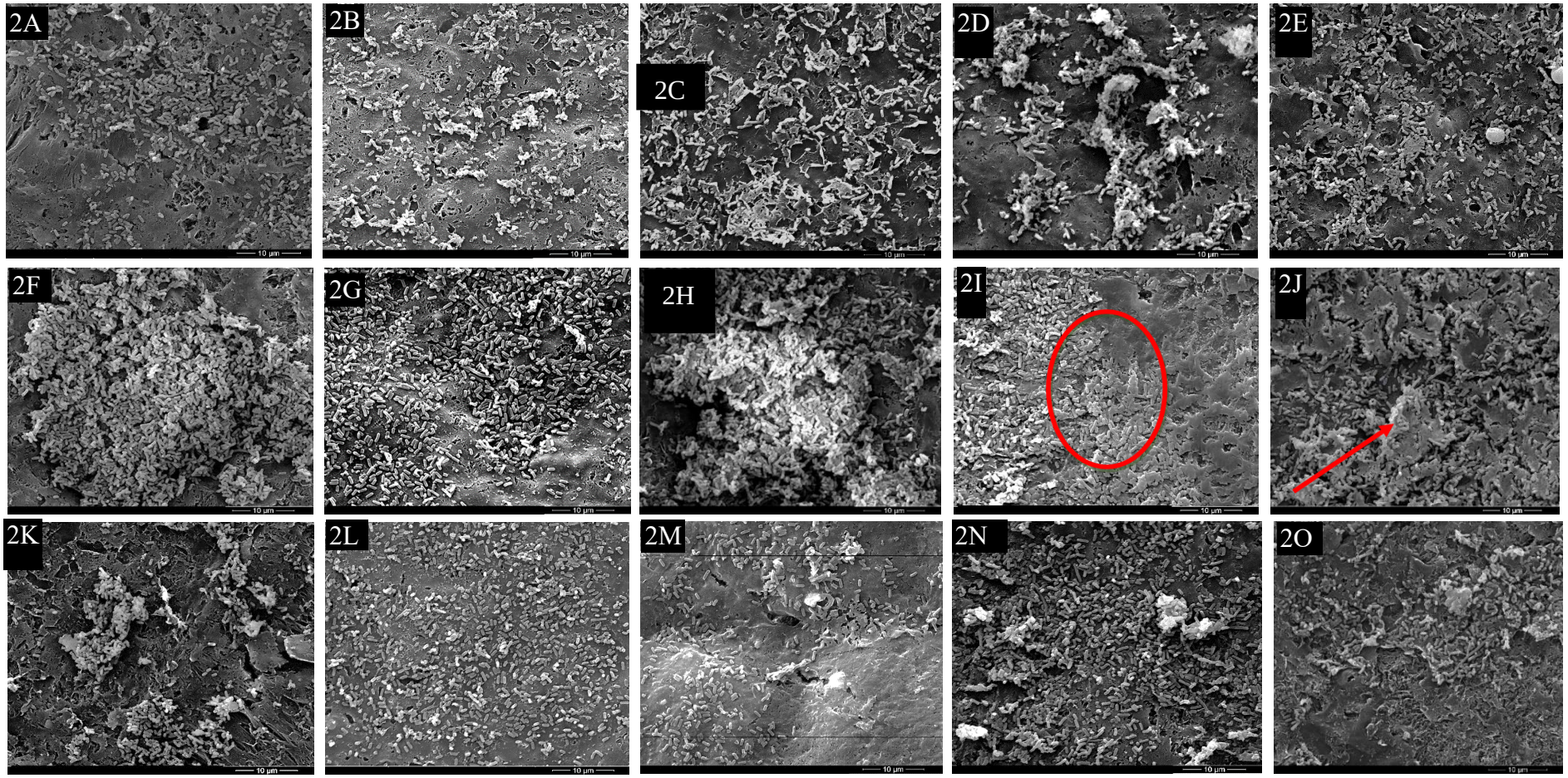

Figure 5.2: Scanning electron microscopy (SEM) of *L. monocytogenes*, lineage I, II and III on the body of 'Royal Gala' apple, across different temperatures, for (2A – 2E), (2A) *L. monocytogenes*, lineage I on day 0, (2B) *L. monocytogenes*, lineage I after14 days at 0.5°C, (2C) 2°C, (2D) 6°C, (2E) at 20°C. Figs (2F-2J) is for lineage II on the body of 'Royal Gala' apple, across different temperatures, where: (2F) *L. monocytogenes*, lineage II on day 0, (2G) *L. monocytogenes* lineage II after14 days at 0.5°C, (2H) at 2°C, (2I) at 6°C, and (2J) at 20°C. Figs. (2K-2O) is for lineage III on the body of 'Royal Gala' apple, across different temperatures, where: (2K) *L. monocytogenes*, lineage III on day 0, (2L) *L. monocytogenes*, lineage III after14 days at 0.5°C, (2M) at 2°C, (2N) 6°C, and (2O) *L. monocytogenes*, lineage III after 14 days at 20°C. Image 2I with an oval shape, and 2J with an arrow, depict an example of a typical biofilm.

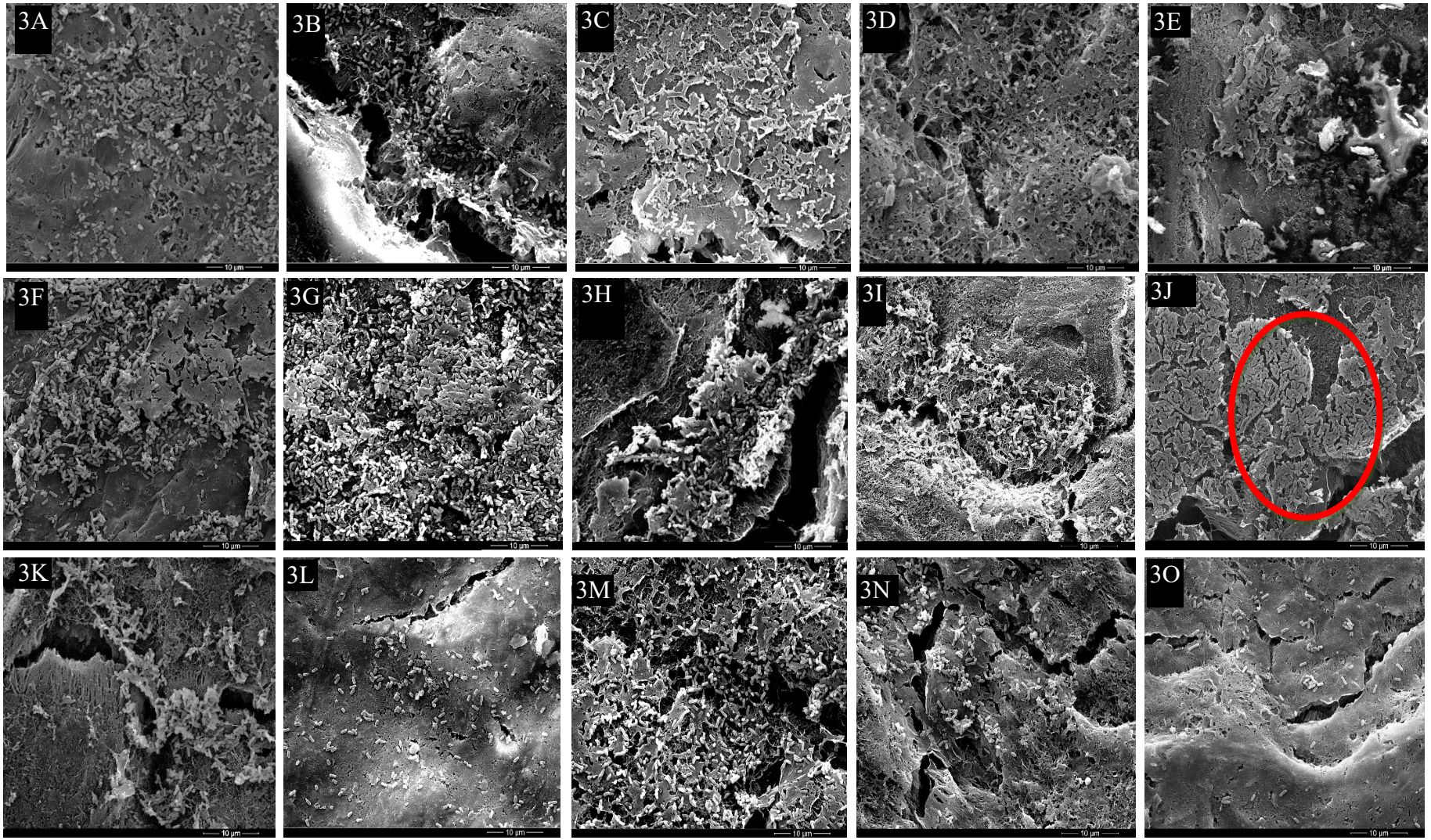

Figure 5.3: Scanning electron microscopy (SEM) of *L. monocytogenes*, lineage I, II and III on the body of 'Scired' apple, across different temperatures, for (3A – 3E), (3A) *L. monocytogenes*, lineage I on day 0, (3B) *L. monocytogenes*, lineage I after14 days at 0.5°C, (3C) 2°C, (3D) 6°C, (3E) at 20°C. Figs (3F-3J) is for lineage II on the body of 'Scired' apple, across different temperatures, where: (3F) *L. monocytogenes*, lineage II on day 0, (3G) *L. monocytogenes* lineage II after14 days at 0.5°C, (3H) at 2°C, (3I) at 6°C, and (3J) at 20°C. Figs. (3K-3O) is for lineage III on the body of 'Scired' apple, across different temperatures, where: (3K) *L. monocytogenes*, lineage III on day 0, (3L) *L. monocytogenes*, lineage III after14 days at 0.5°C, (3M) at 2°C, (3N) 6°C, and (3O) *L. monocytogenes*, lineage III after 14 days at 20°C. Image 3J with an oval shape, depicts biofilm

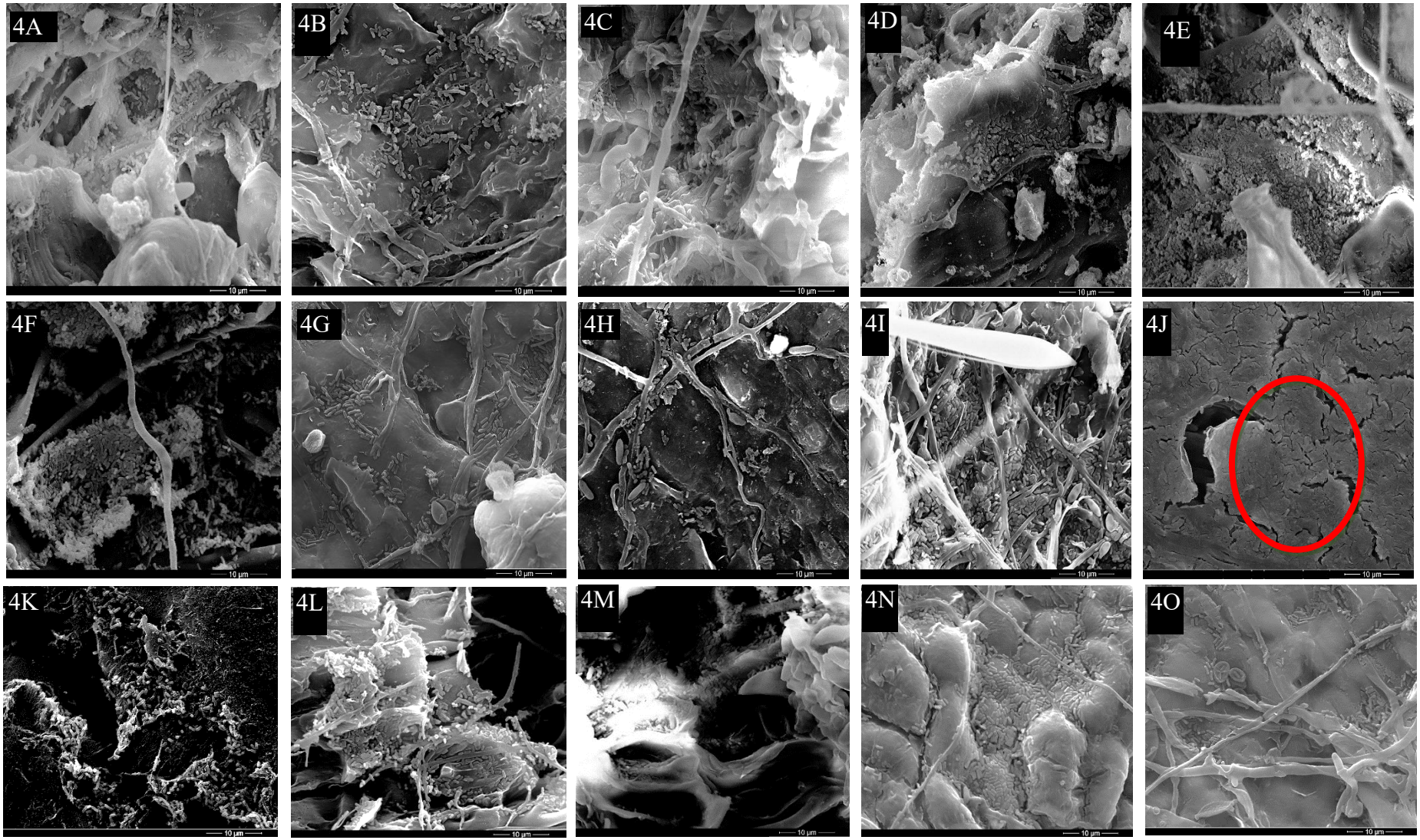

Figure 5.4: Scanning electron microscopy (SEM) of *L. monocytogenes*, lineage I, II and III in the calyx of 'Royal Gala' apple, across different temperatures, for (4A – 4E), (4A) *L. monocytogenes*, lineage I on day 0, (4B) *L. monocytogenes*, lineage I after14 days at 0.5°C, (4C) 2°C, (4D) Figure 5.4: Scanning electron microscopy (SEM) of *L. monocytogenes*, lineage I, II and III in the calyx of 'Royal Gala' apple, across different temperatures, for (4A – 4E), (4A) *L. monocytogenes*, lineage I on day 0, (4B) *L. monocytogenes*, lineage I after14 days at 0.5°C, (4C) 2°C, (4D) 6°C, (4E) at 20°C. Figs (4F- 4J) is for lineage II in the calyx of 'Royal Gala' apple, across different temperatures, where: (4F) *L. monocytogenes*, lineage II on day 0, (4G) *L. monocytogenes* lineage II after14 days at 0.5°C, (4H) at 2°C, (4I) at 6°C, and (4J) at 20°C. Figs. (4K - 4O) is for lineage III in the calyx of 'Royal Gala' apple, across different temperatures, where: (4K) *L. monocytogenes*, lineage III on day 0, (4L) *L. monocytogenes*, lineage III after14 days at 0.5°C, (4M) at 2°C, (4N) 6°C, and (4O) *L. monocytogenes*, lineage III after 14 days at 20°C. Image 4J with an oval shape, depicts biofilm.

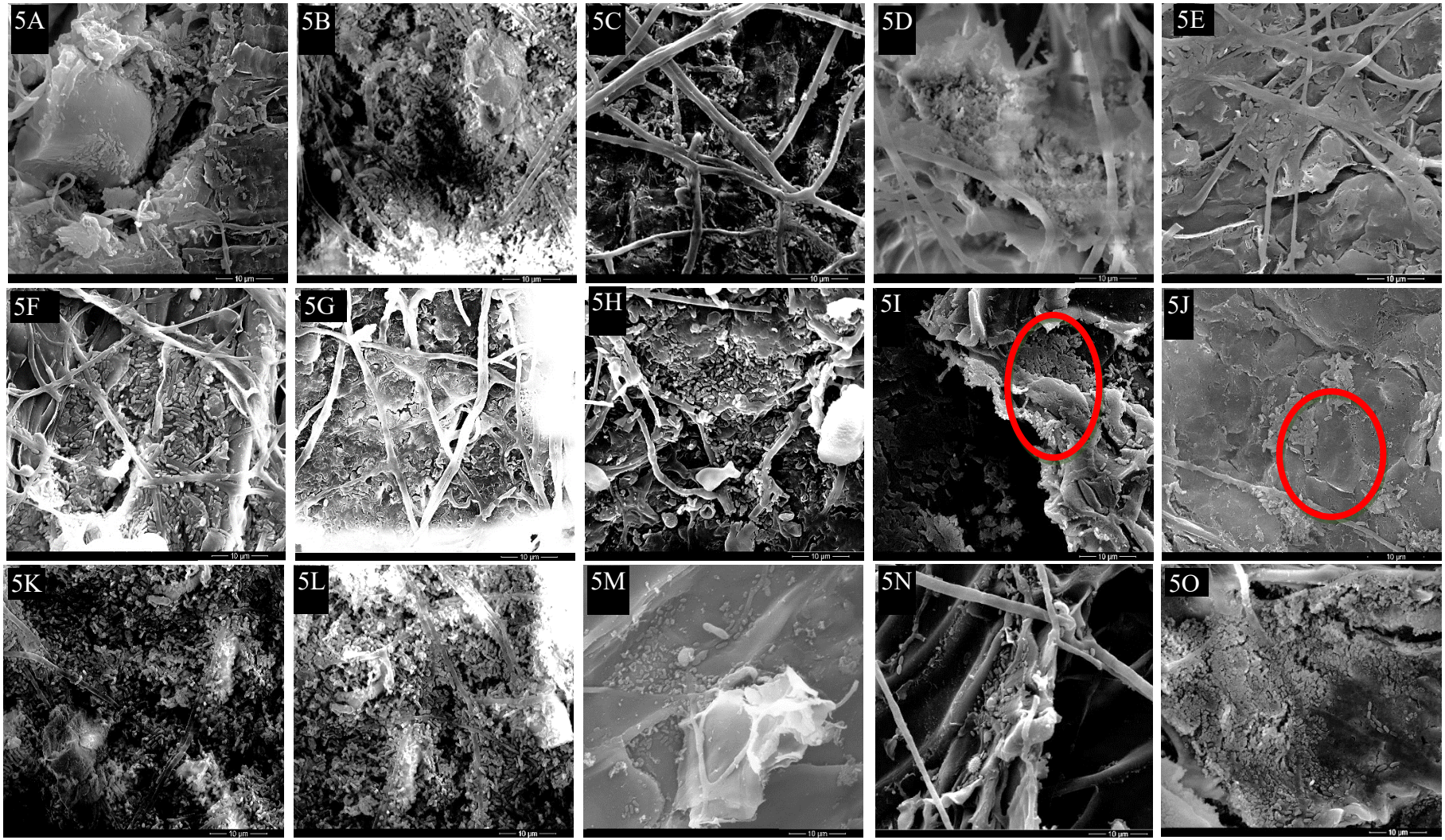

Figure 5.5: Scanning electron microscopy (SEM) of *L. monocytogenes*, lineage I, II and III in the calyx of 'Scired' apple, across different temperatures, for (5A – 5E), (5A) *L. monocytogenes*, lineage I on day 0, (5B) *L. monocytogenes*, lineage I after14 days at 0.5°C, (5C) 2°C, (5D) 6°C, (5E) at 20°C. Figs (5F- 5J) is for lineage II in the calyx of 'Scired' apple, across different temperatures, where: (5F) *L. monocytogenes*, lineage II on day 0, (5G) *L. monocytogenes* lineage II after14 days at 0.5°C, (5H) at 2°C, (5I) at 6°C, and (5J) at 20°C. Figs. (5K - 5O) is for lineage III in the calyx of 'Scired apple, across different temperatures, where: (5K) *L. monocytogenes*, lineage III on day 0, (5L) *L. monocytogenes*, lineage III after14 days at 0.5°C, (5M) at 2°C, (5N) 6°C, and (5O) *L. monocytogenes*, lineage III after 14 days at 20°C. Images 5I and 5J with an oval shape, depict biofilm.

5.3.2.1 Key findings from SEM study:

5.3.2.1a Fungal Interactions

One of the key findings of this study has been the association of lineages with a differing propensity for hyphal proliferation and sporulation in apple storage conditions, which is also probably the reason for high *L. monocytogenes* lineage concentrations in the calyx than on the body (Figure 5.1). Almost all the biofilms in the calyx were attached/ associated with long thread-like structures called fungal hyphae and spores (e.g. Fig. 4H). Fungi and bacteria can form physical and metabolic interdependent associations that have properties distinct from the single entities (Frey-Klett et al., 2011). Such a sophisticated symbiotic relationship is called a biofilm. A biofilm is an association of microorganisms attached to living or non-living surfaces by embedding in a self-produced matrix of extracellular polymeric substances (EPS) (Jamal et al., 2015). A biofilm can combine bacterial and fungal cells or be independent of each other (Frey-Klett et al., 2011).

When the bacteria interact with the fungus, the benefits of a symbiotic relationship are shown to include a possibility of a metabolite exchange for survival, especially the ones neither partner can produce alone (Frey-Klett et al., 2011). When bacteria make biofilms, fungi may provide biotic and nutritional support for their establishment (Frey-Klett et al., 2011; Kjelleberg and Givskov, 2007) and protect against biocides (Alonso et al., 2020). This interspecies interaction also gives resistance to sanitisers like sodium hypochlorite, peracetic acid, and hydrogen peroxide (Yuan et al., 2020). In the apple packhouse facilities, the presence of fungal communities with *L. monocytogenes* indicated the persisting nature of the bacteria in the food processing environments (Tan et al., 2019), showing the importance of eliminating not only the bacteria but fungal communities in the packhouse as well.

5.3.2.1b Biofilm formation in different lineages

SEM in the current study has shown *L. monocytogenes'* ability to make biofilms. Biofilm formed by *L. monocytogenes* poses a severe threat to the safety of ready-to-eat fresh produce as it is difficult for conventional sanitisers to eliminate the bacteria completely. Biofilm formation by *L. monocytogenes* in the food industry environment (e.g. processing equipment) has many advantages, such as physical resistance (against desiccation), mechanical resistance (against liquid streams in pipelines) and chemical protection (against chemicals, antimicrobials and disinfectants used in the industry) (Galié et al., 2018).

Biofilm formation in *L. monocytogenes* is diverse in shapes and sizes, including 3D (Borucki et al., 2003), honeycomb (Colagiorgi et al., 2017; Marsh et al., 2003), knitted chain (Djordjevic et al., 2002), and aggregates (Renier et al., 2011). In the current study, most of the biofilm structures were aggregated (Figures 2I, 3G, 4E, 4I, 5B, 5L), stacked (Figures 2D, 2H, 3H, 5D), knitted (Figures 2E, 5E), and sometimes a mixture of all these shapes (Figures 2J, 4J, 5E, 5G, 5I, 5J). It is important to note that although the SEM images were taken using the exact dimensions and size, the *L. monocytogenes* cells can be shorter than their typical morphology, which may be because of the harsh conditions in the environment or any other stress (Doijad, Barbuddhe, et al., 2015; Somers and Wong, 2004).

A biofilm consists of nucleic acids (Lee et al., 2017), especially extracellular DNA (eDNA). eDNA plays an essential role in the initial adhesion for the attachment during a biofilm formation (Harmsen et al., 2010). It is ubiquitously found in soil, water, and tissue culture (Harmsen et al., 2010). A study on 41 *L. monocytogenes* strains found that eDNA is vital for initial attachment and early biofilm formation (Harmsen et al., 2010).

In Figure 3J, the lineage II *L. monocytogenes* biofilm demarcates with a boundary, possibly due to EPS. Exopolymer substances provides structural complexity, resistance to removal, and antimicrobials (Colagiorgi et al., 2017). Exopolymer substances and water constitute the

extracellular matrix surrounding the biofilm bacteria, including exopolysaccharides, the most vital component of biofilm development (Alonso et al., 2020; Combrouse et al., 2013; Sutherland, 2001). The amount of EPS production depends on the stress and the environmental condition associated with this (Alonso et al., 2020).

As *L. monocytogenes* can survive many adverse conditions, biofilm formation may be an adaptive response to that stress (Lee et al., 2017; Tasara and Stephan, 2006). This adaptive response makes bacterial elimination a significant challenge in the food-processing industries (Giaouris et al., 2014), e.g. more robust biofilms were observed in the milk and milk products, where harsh processing conditions applied during the processing phase, which might have resulted in a stronger biofilm formation (Doijad, Barbuddhe, et al., 2015). SEM of individual lineages (lineage I, II and III) of two cultivars, either on the body or in the calyx, compared with day 0 to day 14, lineage II (Figures 2H, 2I, 2J, 3G, 3H, 3J for body, and 4F, 4H, 4I, 4K, 5F, 5G, 5H, 5I, 5J for calyx) probably have a higher propensity to make biofilms than other lineages. However, the relationship between individual lineages and their ability to make biofilms is unclear (Combrouse et al., 2013). e.g. some authors reported that lineage II strains make more biofilms than lineage I (Borucki et al., 2003), whereas others reported the opposite (Djordjevic et al., 2002; Marsh et al., 2003). Nevertheless, overall, it is suggested that any *L. monocytogenes* strain can make biofilm at a suitable temperature (Kadam et al., 2013), nutrition, pH, salt, the influence of other bacteria (Møretrø and Langsrud, 2004) are probably the reason even lineage III made biofilms in the calyx for 'Royal Gala' (Figures 4L, 4N), and 'Scired' (Figs. 5L, 5O).

5.3.2.1c Temperature responsible for biofilm formation

The criteria to make biofilms for individual lineages of *L. monocytogenes* is complex; e.g. in the current study, most biofilms for individual lineages were made at higher temperatures, i.e. 6°C and 20°C. Temperature plays a pivotal role in the *L. monocytogenes* biofilm formation

(Combrouse et al., 2013; Di Bonaventura et al., 2008). By changing the cell surface, temperature regulates many virulence and environmental genes in *L. monocytogenes* (Siqing et al., 2002). In one study, the growth in the biofilm formation in *L. monocytogenes* increased with an increase in the temperature from 22°C to 37°C (Combrouse et al., 2013; Kadam et al., 2013). Temperature also influences bacterial attachment by flagella and fimbriae (Di Bonaventura et al., 2008). Research also suggested that flagella, not motility, launches the early stages of attachment of *L. monocytogenes* to stainless steel (Vatanyoopaisarn et al., 2000). Temperature-dependent flagellum production was observed at 22°C in *Listeria* spp. during biofilm formation (Di Bonaventura et al., 2008). On the other hand, when the temperature is low, then the *L. monocytogenes* cells undergo cold shock, which results in the modification of cell surface proteins and lipid composition to maintain membrane fluidity homoeostasis, which may help adhesion as an adaptive response against harsh conditions (Lee et al., 2017).

Biofilm formation is influenced not only by temperature but also by the type of fatty acid present in the strain. A particular strain's total fatty acid composition could influence its biofilm-forming capacity (Chihib et al., 2003). While differentiating between a weak, moderate, and strong biofilm-forming strain of *L. monocytogenes* using total fatty acid profiles, it was found that the concentration of iso-$C_{14:0}$, anteiso-$C_{15:0}$, iso-$C_{16:0}$ increased with increasing biofilm-forming strain (Doijad, Barbuddhe, et al., 2015). Also, it was found that the concentration of fatty acid $C_{16:0}$ and $C_{18:0}$ was higher in adherent cells than in non-adherent ones (Gianotti et al., 2008). A lineage-specific study investigating the fatty acid composition could be pivotal in understanding the difference between weak and stronger biofilm. It is important to note that based on environmental stress, a bacteria can change its lipids and fatty acid structure, e.g. from saturated to unsaturated, *cis* to *trans* unsaturation,

branched to non-branched (Dubois-Brissonnet et al., 2016). Also, this could shed light on the presence of more biofilms in lineage II than in any other lineages.

5.4 Conclusions

The microbial analysis (MPN method) concluded that temperatures used in the study had minimal effect on the persistence of individual lineages of *L. monocytogenes*. No growth was found in any lineages of *L. monocytogenes*; however, the bacteria survived. Nevertheless, based on the final bacterial concentrations, as the temperature was static, it looked implausible for the bacteria to grow further. The fresh produce industry relies heavily on low temperatures as a food safety intervention. However, based on the results, one should not concentrate on a low temperature as the only food safety intervention, a systematic whole supply chain approach for the food safety intervention should be followed. As this was one of the initial studies investigating the individual lineages of *L. monocytogenes* and their effect on the body or calyx of apple cultivars, more data is needed to further comment on that.

SEM study showed that the higher persistence of individual lineages of *L. monocytogenes* in the calyx than on the body could be due to strong bacterial-fungal interactions. Hence to understand the bacteria in the calyx, and other hidden structures of apples, bacterial-fungal interactions should be examined in future studies. Based on this study's result in bacterial fungal interactions, this outcome also showed revisiting the risk assessment plans to control *L. monocytogenes* in the apple packhouse facilities or the whole supply chain, as fungal communities in the apples and apple supply chain have often been overlooked. The ability of lineages of *L. monocytogenes* to make biofilms, especially lineage II, is a concern for the apple industry and probably the reason for its more substantial persistence if they enter the packhouse or the supply chain. It should be noted that the SEM study could not explain the structural reasons behind the lower survival of lineage III than I and II, which needs to be focused on in the future.

This study also demands investigating further new research areas to understand the effect of all lineages of *L. monocytogenes* in the apple supply chain, e.g., desiccation stress, fatty acid composition, genetic changes, and bacterial-fungal interactions that could influence bacterial survival.

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Chapter 6: Thesis discussions and industry applications

6.1 Thesis introduction

The literature review (chapter 1) revealed the risks and knowledge gaps regarding the persistence of *L. monocytogenes* in New Zealand's apple supply chains. While examining the effect of long-term (8-10 weeks) and low-temperature (0.5°C) storage practices on the apple supply chain on *L. monocytogenes*, conditions that are crucial for New Zealand horticulture exports, the literature review highlighted that only handful of studies had been done on the persistence or survival of *L. monocytogene*s on various fruits and vegetables, including apples, under different temperatures. Further, the temperatures used in those studies have been unchanged or stable, which is generally impractical in a realistic supply chain, where the temperatures fluctuate ±2°C. The literature also suggested that most of the studies were done on *L. monocytogenes* surrogates (*L. innocua*). This organism's physiology is different from *L. monocytogenes*, hence a need to study the fate of the actual bacteria of concern (*L. monocytogenes*) rather than the surrogate (*L. innocua*), which is non-pathogenic and has different growth patterns to *L. monocytogenes* (Omac et al., 2015) is crucial.

While considering the fate of *L. monocytogenes* in the domestic supply chain, e.g. the distribution centre (DC), and grocery stores, the literature showed critical gaps in the risks, including the high dynamic temperatures. Each step along the supply chain could contribute to foodborne pathogens' potential contamination and growth. However, there needs to be more information and data associated with contamination in every step of the fresh produce supply chain, so it is complicated to assess the associated risk. Hence more research is needed to assess the persistence of *L. monocytogenes* at the domestic supply chain level (Townsend et al., 2021).

While looking into the effect of individual lineages of *L. monocytogenes* on apples, preliminary studies investigate the ecology, distribution, and persistence of *L. monocytogenes.* Similar results were received when the persistence of *L. monocytogenes* with apples was investigated in storage and bacterial attachment studies.

The above knowledge gaps in the literature review formed the basis of challenge studies performed in the thesis:

- The effect of dynamic temperatures on *Listeria monocytogenes* in the international supply chain (Chapter 2)
- The effect of dynamic temperatures on *Listeria monocytogenes* in the domestic apple supply chain (Chapter 3)
- The effect of storage temperatures on the survival of three lineages of *Listeria monocytogenes* on apples (Chapter 4)
- The effect of commercial storage temperatures on the survival and attachment of three lineages of *Listeria monocytogenes* on two apple cultivars (Chapter 5)

The chapters mentioned above disproved the hypothesis that as the temperature in the apple supply chain is variable/dynamic in nature, the risk of *L. monocytogenes* population should increase under those conditions. In point of fact, the thesis proved the minimal risk from *L. monocytogenes* contamination of apples, which is useful for food safety of ready to eat product like apple.

6.2 Key discussions

6.2.1 Dynamic and static temperatures did not result in growth of L. monocytogenes

The challenge studies in chapters 2, 3 and 4 revealed that under the dynamic and static temperatures considered, no growth of *L. monocytogenes* was observed on apple surfaces throughout the challenge studies. For dynamic conditions, *L. monocytogenes* concentrations in the international sea freight (New Zealand to USA and Europe) and domestic supply chain

declined. A similar result was observed under static temperatures as well. This result proved that under the dynamic and static conditions used in this study, the temperature played a minimal role in the survival of *L. monocytogenes*, a result that with the previous research (Sheng et al., 2017). This study also included shelf-life (ambient) and display cabinet temperatures (20°C ± 2ºC); even those high temperatures resulted in the decline by a further 1 $\log_{10}$ MPN/apple of the *L. monocytogenes*.

It is important to note that long-term or short-term low-temperature storage studies were performed. However, the concentration of *L. monocytogenes* decreased, but it did not die off completely. Weibull inactivation kinetics showing shape and scale factor indicated that weak populations of *L. monocytogenes* were destroyed, leaving behind survivors that were stress resistant. The survival of stress-resistant bacteria established that although a strict low-temperature supply chain is important, it should not be considered the only risk mitigation strategy to control *L. monocytogenes*.

6.2.2 L. monocytogenes lineage III has a lower survival rate than lineage I and II

Chapters 4 and 5 confirmed that no growth was observed in the study but sought to understand the efficacy of individual lineages of *L. monocytogenes* under different temperatures (0.5, 2, 6, 20°C). MPN results showed that lineage III had a lower survival rate than lineages I and II. SEM could not explain any reasoning behind the lower survival of lineage III than I and II. As all the isolates of each lineage used in the study have been derived from horticultural sources, the challenge studies revealed lineage I and II survive better in harsh and stressful environments than lineage III. The survivability of lineages I and II are hypothesised to be due to tolerance to limited nutrition (Hingston et al., 2017) and the presence of extrachromosomal genes (Orsi et al., 2008).

6.2.3 MPN detection method is the preferred method over qPCR

Compared with the MPN method, chapter 4 showed that a rapid detection method like qPCR gave higher *L. monocytogenes* concentrations than expected, possibly due to the reaction reading of both dead and alive bacteria. qPCR read dead and live bacteria gave valuable information on the need to be vigilant when using qPCR for bacterial quantification. This might be the reason why qPCR is not popular for bacterial quantification, and MPN is still regarded as a reliable methodology. However, rapid genetic quantification through mRNA is promising and it only targets viable cells. As mentioned in chapter 4, there are reports on developing nucleic acid amplification-based assays for *L. monocytogenes* targeting RNA (Blais et al., 1997; Herman, 1997; Klein and Juneja, 1997; Norton and Batt, 1999).

6.2.4 The requirement to investigate the carrying capacity of L. monocytogenes on apples

The *L. monocytogenes* lineages were also used to investigate the carrying capacity of apples at different temperatures in chapter 4. High and low inoculum concentrations for individual lineages performed under different temperatures also resulted in no growth of *L. monocytogenes*. The result on carrying capacity proved that no growth occurred in any of the lineages, even with a low inoculum, means the carrying capacity had already been reached. This showed that the carrying capacity of apples is lower than the low inoculum (~6 $\log_{10}$ MPN/apple) used in the study. To find out the growth potential of *L. monocytogenes*, carrying capacity should be looked at in the future for each apple cultivar.

6.2.5 Open and closed calyx apple cultivars show no difference in the L. monocytogenes survival

The apple cultivars used in the studies were representative of open and closed calyx cultivars, and the results from all the challenge studies proved that open or closed calyx cultivars used in this study pose no difference for *L. monocytogenes* survival. Regardless of the cultivar, the survival of *L. monocytogenes* was higher in the calyx than on the skin (body) of the apple.

This is an important finding in light of the caramel apple outbreak in 2015 in the USA. During that outbreak, inserting the stick in the apple was an important factor as it drove the organism into the apple core, resulting in cellular damage to the apple and creating conditions that allowed *L. monocytogenes* to grow (Glass et al., 2015). The current study proved that the tendency of *L. monocytogenes* survival was higher in the calyx of the apple than on the skin, meaning that the calyx is most likely the point of bacterial harbourage in apples. This may be relevant in understanding the pathway of *L. monocytogenes* contamination in the caramel apple outbreak. Putting a stick through the contaminated calyx could result in cross-contamination and internalise the bacteria in the apple flesh, which might have happened in the caramel apple outbreak in 2014/2015. It also demonstrates greater risks to consumers who eat the apple core rather that those who only consume the flesh.

6.2.6 Scanning electron microscope (SEM) showed evidence of a bacterial-fungal relationship

The SEM study in chapter 5 showed that the presence of a higher concentration of every *L. monocytogenes* lineage in the calyx than the body was possibly due to a bacterial-fungal symbiotic relationship, the critical finding of the SEM study. SEM study also proved that the bacterial-fungal relationship resulted in the formation of strong biofilms in the calyx, especially at 6 and 20°C rather than 0.5°C. There was an association of *L. monocytogenes* lineages with a differing propensity for hyphal proliferation and sporulation in apple storage conditions. This result of the higher attachment of *L. monocytogenes* in the calyx than on the body could have resulted in the caramel apple listeriosis outbreak, in which a stick was inserted into the hidden parts of the apple flesh. However thorough research is needed to further investigate this.

6.3 Industry applications

6.3.1 Sea freight temperature management in the international supply chain

This research is the first-ever food safety study on dynamic (realistic) apple supply chain temperatures to the author's knowledge. This multi-faceted study followed the fate of *L. monocytogenes* in open and closed calyx cultivars in the international supply chain. The survival of *L. monocytogenes* in these conditions was demonstrated when sending apples from New Zealand to the USA or Europe.

This study showed no significant difference ($P > 0.05$) between the persistence of *L. monocytogenes* in open and closed calyx cultivars. The data gathered in this exercise disproved the common perception that *L. monocytogenes* grows better in open calyx cultivars than in closed, and gave the apple industry more confidence in their produce.

L. monocytogenes grows at variable temperatures (Santos et al., 2019). Previously, no data was available on the impact of the dynamic temperatures experienced in the apple industry. The data from the current study suggested that *L. monocytogenes* did not grow under dynamic conditions, from 6°C to 0.5°C, giving apple packhouse operators confidence in the temperatures that they work with.

After sea-freight temperatures, simulated shelf-life studies were also performed where the fruit at 20°C was analysed for *L. monocytogenes* persistence. The result suggested that *L. monocytogenes* did not grow even at shelf-life temperatures. It provided good baseline information for retailers who display their apples at room temperature and consumers when they store them at ambient.

6.3.2 Domestic supply chain management

The temperature in the domestic supply chain is highly dynamic and changes significantly due to several steps involved e.g. trucking, DCs, grocery stores, and display cabinets.

Generally, when fresh produce moves from the packhouse to the retail market, wholesalers and retailers in the DCs and grocery stores need more facilities to meet different temperature requirements for every commodity. Depending on the facilities, inventory managers decide how produce will be handled, and priority is given to temperature-sensitive products like leafy greens, and tomatoes. This is probably the reason why resilient fresh fruit like apples receive the most significant temperature abuse (Paull, 1999). However, even at those highly fluctuating temperatures, the study showed that *L. monocytogenes* did not grow. This outcome gave the wholesalers and retailers including transportation and distribution centre decision-makers more confidence in their operating temperatures, .

6.3.3 Rapid detection methods for L. monocytogenes

The study compared two quantitative methods for individual lineages of *L. monocytogenes* detection: conventional and molecular biology (qPCR).

The result indicated that while the conventional detection method was time-consuming (3-4 days), the qPCR methodology gave results which combined live and dead bacterial concentrations. The higher concentration results from the qPCR method could be due to its inability to distinguish between living and dead cells or the qPCR could be detecting VBNC cells.

Several countries like Singapore and Malaysia follow FSANZ guidelines, which means that RTE foods like apple need to have *L. monocytogenes* counts of fewer than 100 CFU/g. However, the USA has zero tolerance for *L. monocytogenes* detection, which is significant for countries like New Zealand with horticulture-based economies. Many other countries also use tight regulations against *L. monocytogenes* on apples. Finding the exact microbial quantification onshore will be the only way in the future, as it will help achieve ideal market access goals for each country. Many commercial laboratories still use multiplex PCR, which

makes the presence and absence of the bacteria of concern. These tests, however, are valuable in their own right (e.g. finding presence/absence for zero tolerance countries like the USA). However, these results do not give the quantified bacteria levels, which is more important for the exporters or retailers if they have any problem with their apples.

This study considered options for rapid quantitative detection methodologies for *L. monocytogenes* and suggested a mRNA-based method as the assay is relatively faster and more sensitive. Success in finding a rapid and inexpensive method will benefit every grower from the technique, especially when their products are on hold due to market access restrictions. In conjunction with that, identifying an actual lineage of *L. monocytogenes* could be very helpful in finding the sources of microbial load if a problem occurs, even better if a sequence type could be identified.

6.3.4 Bacterial-fungal relationship in apples

The study was done on two apple cultivars to quantify individual lineages of *L. monocytogenes.* The MPN study showed that lineage I and II fared better than lineage III for all the temperatures. The scanning electron microscopy (SEM) study gave the same outcome. Lineage II can attach to the apple surface and make biofilms, which is probably why lineage II is more prevalent in food and food processing environments (Orsi et al., 2008).

As in the studies in other chapters, *L. monocytogenes* survived better in the calyx than on the body ($P < 0.05$), possibly due to the bacterial-fungal relationship in the calyx. When the apples pass through packhouse processes, sanitisers cannot clean every part of the apple, possibly allowing fungal cells to proliferate. Hence the fungal communities always dominate the calyx of an apple (Yuan et al., 2020). If *L. monocytogenes* contamination happens and the bacteria reaches the calyx, it will be challenging to eradicate them. Some studies also suggested bacterial-fungal interactions could resist sanitisers (Yuan et al., 2020).

This result is significant for the apple packhouse operators, as this research showed possible *L. monocytogenes* and fungal interaction. The packhouse operators could re-think their risk-assessment strategy and possibly include interventions to decrease the fungal proliferation and ultimately decrease *L. monocytogenes* biofilm formation.

6.4 Future directions

6.4.1 Development of a Risk assessment model

The data generated from this thesis is ideal to be included in a quantitative microbial risk assessment (QMRA) model for *L. monocytogenes* for both international and domestic supply chains. As New Zealand is an apple exporting country, with more than 20 varieties being exported, every apple has its postharvest storage and processing requirements. Developing individual models from each of the varieties could be beneficial for encountering any food safety issues in the future.

6.4.2 Future-proofing market access

As the study based on the international supply chain was the first-ever study on the actual dynamic temperatures and *L. monocytogenes* survival, more data is needed to future-proof all the market access aspects of a supply chain. Generating more data leads to a better understanding of the behaviour of *L. monocytogenes* when the apples are sent from New Zealand to different parts of the world.

6.4.3 Focus on food safety issues in the domestic supply chain

Packhouses, transportation, distribution centres, and retail markets all have a role to play in the successful and pathogen-free supply chain. There has been very little or no data available before the current thesis. More data must be generated across the country so all the environmental variations can be mapped out.

6.4.4 Characterising background microbial community

The research suggested that *L. monocytogenes* did not grow on apples, but inactivation kinetics also suggested that the bacteria could survive. An in-depth study of the microbial community that may influence *L. monocytogenes* positively or negatively could be crucial.

6.4.5 Relative Humidity: a critical component for L. monocytogenes

The temperature has been the primary component studied in the current thesis. Another component that needs attention is the relative humidity (RH) in the apple supply chain which should be examined in the future. Relative humidity (RH) affects water loss from plant issues (Likotrafiti et al., 2013) and a reduction in the RH decreases the survival of *L. monocytogenes*, and vice versa (Redfern and Verran, 2017). Studies suggest RH to be interdependent of temperature, but RH can only prevent water loss when the temperature of the fresh produce is close to ambient (Paull, 1999). Change in the RH could lead epiphytic bacteria like *S. enterica* to undergo stress, and studies suggested a rapid bacterial reduction when the RH decreased from 100 to 60% (Brandl and Mandrell, 2002).

Temperatures in the apple supply chain are highly dynamic and change rapidly, particularly for the domestic market. Temperature management alone is not enough to control the microbial colonisation of *L. monocytogenes*. As the current thesis concentrated on temperature, RH case studies and looking into matrix studies with different temperatures on apples may shed light on the *L. monocytogenes* survival when apples go from New Zealand to countries with high humidity.

6.4.6 Developing RNA-sequencing methodologies for the quantification of L. monocytogenes

The current thesis identified that while culture-based methodologies and qPCR have their place in quantifying *L. monocytogenes*, new and more sensitive methodologies are required.

While qPCR can only quantify known genes, RNA sequencing can quantify known and novel genes using next-generation sequencing (NGS).

6.4.7 Apple's skin physiology as a vital indicator of the bacteria attachment

The skin of an apple comprises the cuticle, epidermis, and hypodermis. The cuticle provides a protective barrier for water transport, gas exchange, and pathogen defence. The skin thickness of an apple differ in every cultivar. It also differs in the same cultivar across different seasons (Homutova and Blazek, 2006).

The thesis studied the attachment of *L. monocytogenes* on the skin of two apple cultivars. However, there is limited literature on the attachment mechanism of the bacteria for the different New Zealand cultivars, which could be beneficial to categorise the bacterial attachment risk on the apples based on their skin properties (if any).

Also, it would be worthwhile examining the ability of apple skin to support biofilm of other microorganisms. The toxicity of the skin or microstructure may discourage colonization.

6.4.8 Understanding the fungal-Listeria monocytogenes relationship in the calyx of apple for the formation of biofilm

The thesis showed that one crucial reason behind the better survival of *L. monocytogenes* in the calyx than on the body of an apple may be the bacterial association with the fungal community. However, there is not enough research to understand their relationship, e.g., which type of fungal community protects and enhances the survival of *L. monocytogenes* and helps them make a biofilm and the mechanisms for this.

6.4.9 Impact of waxed apples on the fate of L. monocytogenes

Waxes are used to make apples shiny. Shellac and Carnuba wax are approved as food additives by FSANZ (Food Standards Australia and New Zealand) (APAL, 2016). These waxes are not only approved in Australia and New Zealand, but also in the USA, UK, and

Europe (APAL, 2016). Waxing apples helps retain moisture (APAL, 2016), preventing the apples from shrivelling. However, *Listeria* species in the apple packing facility are commonly found in the waxing area (Ruiz-Llacsahuanga et al., 2021b). They can grow on the equipment's surfaces as biofilms and become protected by wax residues (Ryser et al., 2019). As waxing is considered the last step of apple processing in the packhouse, it can impact the microbial population in apples (Ryser et al., 2019). Wax coating could help the survival of *L. monocytogenes* on apple surfaces (Macarisin et al., 2019). The fate of *L. monocytogenes* should be looked at with waxed apples under storage, as there are gaps in understanding the microbial risks associated with the postharvest practices of tree fruit production (Macarisin et al., 2019). This should be included with the bacterial attachment study to find out how *L. monocytogenes* attached to waxed surface, in comparison to the unwaxed one.

6.4.10 Impact on quality attributes of apple cultivars for L. monocytogenes

By 2018, the New Zealand apple industry was ranked the most competitive industry in the world due to the varieties it produces (Sofkova-Bobcheva et al., 2021). New Zealand grows about 27 different apple varieties, including 'Braeburn', 'Royal Gala', Envy™, Jazz™, and Dazzle' (Sofkova-Bobcheva et al., 2021). Every apple cultivar has its harvest and storage regimes. For example, immediately after harvest, the apple variety Braeburn must follow a step-down cooling process to mitigate the risk of internal browning , where the apple variety transitions from higher temperature to lower temperature. Every cultivar, when exported, has its levels of soluble solids (°Brix). For example, Braeburn going to Europe has a °Brix value of 10.5, while for Envy™ it is 15 (Sofkova-Bobcheva et al., 2021). Different attributes of different apple varieties have not been assessed with the pathogen of concern, *L. monocytogenes* and it would be worthwhile to assess them.

6.4.11 Targeting other exporting countries to see temperature variations

New Zealand has a very small domestic market; hence it has excess production enabling it to export to roughly 65 international destinations (Walker et al., 2015). Before 1990, New Zealand's main exporting markets were the USA and UK. Nevertheless, things changed after food safety outbreaks in the UK, forcing New Zealand to look for new exporting destinations (Walker et al., 2015). New Zealand now exports apples to China, Vietnam, Taiwan, Thailand, India, Belgium, Russia, the USA and UK (Freshfacts, 2020). The current thesis concentrated on two international destinations, the USA and Europe. However, as every country's environmental conditions are different, the survival of *L. monocytogenes* in those specific environmental conditions is not yet quantified and should be looked into in the future, especially in tropical markets with possible high temperatures that were not covered in the current studies.

6.4.12 Different inoculation methodologies

The current thesis investigated the survival of *L. monocytogenes* using the wet inoculation method on apples. Another inoculation methodology that needs further investigation is dry inoculation (Girbal et al., 2021). Although the inoculation method effect varies by fresh produce, dry inoculation may simulate a more realistic cross-contamination route in agricultural settings, which could provide valuable results (Girbal et al., 2021). However, for *L. monocytogenes*, because of its ubiquitous, there are so many potential cross-contamination The dry inoculation method is unexplored in fresh produce like apples, with few studies on the survival of *L. monocytogenes*. However, although temperature played a significant role, *L. monocytogenes* studies on carrots, tomatoes, and cauliflower showed an increase in bacterial growth for the wet inoculation method (versus the dry method) (Girbal et al., 2021). For *Escherichia coli* O157:H7 on hazelnuts, when the survival of the bacteria was assessed

using 7 log CFU, the survival was significantly reduced under dry inoculation compared to wet inoculation (Feng et al., 2018).

6.4.13 Transportation delays during current complex shipping scenarios post-covid

Although there is no evidence that Covid-19 is transmitted through food, it had a massive impact on the supply chain, and the international food trade is dependent supply chain (Kahramanoğlu et al., 2021). During the pandemic, many countries shut down their whole economy to slow the spread of Covid-19 (FAO, 2020). Lockdowns resulted in the closing of markets, transport delays (especially sea freight), port closures, and logistic hurdles, which affected import/export (Kahramanoğlu et al., 2021). As New Zealand is not on a general global shipping route, Covid-19 has created significant challenges for New Zealand's international supply chain, as sea freight carries 99% of the country's trade by volume and 80% by value (Salmond and Richards, 2021). As a result of port delays around the world, New Zealand's shipping schedule reliability fell from 80% pre-pandemic to 6% post-pandemic (Salmond and Richards, 2021). This has resulted in an extended shipping schedule for fresh produce . If fresh produce like apple has a pathogenic bacterial load due to cross-contamination, the longer shipping delays could give time for the bacteria to acclimate. There must be challenge studies looking into shipping delays and food safety concerns. It is important to note that shipping in general has slowed in order to reduce fuel costs and environmental impact. This is a challenge right across the food industry.

6.4.14 Organic vs inorganic apples on the fate of L. monocytogenes

Organic and conventional apples have different agricultural practices, and the apple varieties from both agricultural practices could behave differently during bacterial adhesion (Sheng et al., 2017). A study on conventional and organic granny smith apples during storage found no significant difference in the survival of *L. monocytogenes* (Sheng et al., 2017). However, whether these results hold during dynamic conditions when sending the apples from New

Zealand to other parts of the world (or even domestic) is unknown. Also, another study found that the bacterial composition of conventional apples had more potential for harbouring foodborne pathogenic bacteria than organic apples. The microbiome of organic apples included *Lactobacillus,* a probiotic bacterium (Wassermann et al., 2019). Investigating the antioxidant activity of such lactic acid bacteria could contribute to differences in *L. monocytogenes* adhesion organic and conventional apples (if any).

6.4.15 Finding the carrying capacity of L. monocytogenes in apples

Carrying capacity is the maximum number of individuals (*L. monocytogenes* cells in this case) that an environment or a host can sustain (Wein et al., 2018). As the study on the individual lineages suggested no growth, individual lineages may have reached the carrying capacity. An example of cucumbers also suggested no growth in the *Salmonella* population at a given temperature, suggesting the bacteria reached carrying capacity (Bardsley et al., 2019). In the current study on apples, multiple low inoculum levels were not tested. Further research should investigate whether low inoculum levels influence the growth and survival of *L. monocytogenes* in apples or no growth on apples at all.

6.4.16 SEM vs environmental SEM (ESEM) for visualisation

When studying the biofilm composition, it is crucial to visualise the biofilm in its most delicate details; hence SEM is used (Relucenti et al., 2021). The current study used conventional SEM, which is the best method for visualising biofilm structure and morphology. However, as the bacteria are fixed in the conventional SEM, they are not in their "living natural" state. In ESEM, biofilms could be visualised without pre-treatment, thus saving their integrity and without any dehydration and loss of mass (Bossù et al., 2020; Relucenti et al., 2021). ESEM has some drawbacks, like a lack of conductivity-lowering resolution (Relucenti et al., 2021). However, it would be worthwhile to investigate the best electron microscopy method to visualise biofilms on the apples.

6.4.17 PMA qPCR to quantify VBNC

In the current chapter and some other chapters as well, the concept of VBNC is indicated. The VBNC is a survival strategy of the bacteria that cannot grow on routine culture media but is still alive and metabolically active (Truchado et al., 2020; Zhao et al., 2017). Staining techniques have been used to differentiate between dead and VBNC cells, assuming that dead cells have their membrane compromised. However, not all dead cells have their membrane compromised, and as a result, this method could lead to an overestimation of the VBNC number (Truchado et al., 2020). A qPCR in combination with a photoreactive dye such as propidium monoazide (PMA) is an effective method to quantify VBNC (Nocker et al., 2006). PMA penetrates the dead cells, compromising membrane integrity and binding to the DNA, preventing subsequent PCR amplification (Nocker et al., 2006). Optimising PMA qPCR for *L. monocytogenes* quantification on apples could be beneficial in answering the question of whether the VBNC state of the bacteria could be an issue in the horticulture industry.

The thesis provided valuable information on the survival of *L. monocytogenes* at different temperatures and scenarios. This thesis will be helpful for the New Zealand apple industry and wider tree crop industries to understand bacterial risk mitigation strategies, and it will provide added food safety assurance for the apple producers.

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